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INTERACTION BETWEEN BRK AND HER2 IN BREAST CANCER

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INTERACTION BETWEEN BRK AND HER2 IN BREAST CANCER

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INTERACTION BETWEEN BRK AND HER2 IN BREAST CANCER

A

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DOCTOR OF PHILOSOPHY

by

Midan Ai, M.S.

Houston, Texas

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DEDICATION

To my parents, my parents in law, my husband, my lovely daughter and son,
Vivian and Leo and all my family members and friends in China and in USA for their
unconditional love

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INTERACTION BETWEEN BRK AND HER2 IN BREAST CANCER

Publication No. _____

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Breast tumor kinase (Brk) is a nonreceptor protein-tyrosine kinase that is highly expressed in approximately two thirds of breast cancers but is not detectable or is expressed at very low levels in normal mammary epithelium. Brk plays important roles in promoting proliferation, survival, invasion, and metastasis of breast cancer cells, but the mechanism(s) of which remain largely unknown. Recent studies showed that Brk is frequently co-overexpressed with human epidermal growth factor receptor-2 (HER2) and is physically associated with HER2 in breast cancer. The mechanism needs to be determined. In my studies, I found that high expression of HER2 is correlated with high expression of Brk in breast cancer cell lines. Silencing HER2 expression via RNA interference in HER2 over-expressed breast cancer cells resulted in Brk protein decrease and overexpression of HER2 in HER2 low-expressed breast cancer cells up-regulated Brk expression. The mechanism study indicated that overexpression of HER2 increased Brk protein stability. Brk was degraded through a Ca^{2+} -dependent protease pathway involving calpain and HER2 stimulated Brk expression via inhibiting calpain activity. Calpastatin is a calpain endogenous inhibitor and the calpain-calpastatin system has been implicated in a number of cell physiological functions. HER2 restrained calpain activation via up-regulating calpastatin expression and HER2 downstream signaling, MAPK pathway, was involved in the regulation. Furthermore, silencing of Brk expression by RNA interference in HER2-overexpressing breast cancer cells decreased

HER2-mediated cell proliferation, survival, invasion/metastasis potential and increased cell sensitivity to HER2 kinase inhibitor, lapatinib, treatment, indicating that Brk plays important roles in regulating and mediating the oncogenic functions of HER2. The Stat3 pathway played important roles in Brk mediated cell survival and invasion/metastasis in the context of HER2-overexpressing breast cancer cells. However, transgenic mice with inducible expression of constitutively active Brk (CA) in the mammary epithelium failed to develop malignant change in the mammary glands after Brk induction for 15 months which indicated that expression of Brk protein alone was not sufficiently to induce spontaneous breast tumor. Bitransgenic mice with co-expression of HER2/neu and inducible expression of Brk in the mammary epithelium developed multifocal mammary tumors, but there were no significant difference in the tumor occurring time, tumor size, tumor weight and tumor multiplicity between the mouse group with co-expression of Brk and HER2/neu and the mouse group with HER2/neu expression only.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell mediated cytotoxicity
ALLN	N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine
Brk	Breast tumor kinase
BSK	Brk substrate
CDK	Cyclin-dependent kinase
CHX	Cycloheximide
CIS	Carcinoma in situ
EDTA	Ethylenediaminetetraacetic acid
EGFR	The epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ER	Estrogen receptor
erbB2	Erythroblastic leukemia viral oncogene homolog 2
FBS	Fetal bovine serum
DCIS	Ductal carcinoma in situ
Dox	Doxycycline
ECD	Extracellular domain
EMT	Epithelial mesenchymal transition
GAPs	GTPase-activating proteins
HER2	Human epidermal growth factor receptor 2
HSP90	Heat shock protein 90
IDC	Invasive ductal carcinoma
IGF	Insulin-like growth factor
IVIS	In vivo imaging system

KO	Knock out
LCIS	Lobular carcinoma in situ
MAPK	Mitogen-activated protein kinases
MET	Mesenchymal epithelial transition
MMTV	Mouse mammary tumor virus
MTB	Mouse tumor biology
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3' -kinase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTK6	Protein tyrosine kinase 6
RFU	Relative fluorescence units
rTA	Tetracycline transactivator
rtTA	Reverse tetracycline transactivator
Sam68	Src-associated substrate in Mitosis of 68 kDa
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SH3	Src homology 3
Sik	Mouse Src-related intestinal kinase
SLM-1	Sam68-like mammalian protein-1
SLM-2	Sam68-like mammalian protein-2

SOCS3	Suppressor of cytokine signaling 3
STAP-2	Signal-transducing adaptor protein-2
STAT3	Signal transducer and activator of transcription 3
Tg	Transgenic
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WAP	Whey acidic protein

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Breast cancer is listed as No. 1 of the three most common cancers including lung cancer and colorectal cancer among American women. It is also an alarmingly common and serious health concern for women worldwide, therefore much research target the breast cancer. Our research focus on the breast tumor kinase (Brk), a nonreceptor tyrosine kinase originally cloned from a metastatic breast tumor. Brk is overexpressed in most breast tumors, particularly in advanced invasive and metastatic tumors, but is not detectable in normal breast epithelial cells. Co-overexpression of Brk and HER2 is universally found in archived human breast cancer specimens but the mechanism of co-overexpression remains mysterious. Brk's role in breast tumorigenesis has been extensively studied, however, the interaction between Brk and HER2 and how Brk is involved in HER2 induced oncogenic functions and tumorigenesis has seldom been studied. The background provided here covers the broad area of breast cancer, EMT, calpain-calpastatin system and transgenic mouse models. In addition, a review of the literature on the roles of HER2 and Brk in the breast cancer development will let us better understand the interaction between Brk and HER2 and the Brk's involvement in the oncogenic process mediated by HER2.

BREAST CANCER

Breast cancer accounts for 23% of all cancers in women worldwide. In 2012, it was estimated that about 226,870 new cases of invasive breast cancer and 63,300 new cases of carcinoma in situ (CIS) would be diagnosed in women in the United States, and among them 39,510 women would die from breast cancer [1].

Risk Factors

Reproductive risk factors have been correlated to an increased risk of breast cancer. The reproductive risk factors include ages at menarche, menopause, and first-term pregnancy [2]. Generally, women who start menstrual period earlier have higher risk than those start the period later. On the contrary, women who have later menopause are at higher risk than women who have earlier menopause. Other factor like getting a full-term pregnancy before the age of 30 would decrease the risk. The estrogen-progestin is able to stimulate cell proliferation, patients who receive postmenopausal estrogen-progestin replacement therapy especially those who use it for more than 5 years have been shown to have the risk of breast cancer increased up to 50% [3, 4]. Oral contraceptives increase the risk of breast cancer for the same reason [5, 6]. Genetic predisposition is another high risk factor, Family history of breast cancer contribute to 20 percent of women with this disease and among which 5 to 10 percent have an alteration in the breast cancer susceptibility gene either BRCA1 or BRCA2 [7-9]. Breast cancer is also an aged problem because most cases occurring in women over the age of 60.

There are some factors like early or multiple pregnancies, lactation, physical activity and avoidance of alcohol that reduce the risk of breast cancer [2, 3, 10, 11]. Multiple pregnancies decrease the risk by 20% and a full term pregnancy before the age of 30 years reduce the risk by 30%. Taking breast cancer chemoprevention medicine such as tamoxifen decreases the risk by about 40% for high-risk women. Breast-feeding offers a small but effective protective function. Women who have a

healthy diet with adequate amounts of fruits and vegetables, olive oil, and soy products were less likely to have breast cancer than women who have unhealthy diet.

Classification

According to the histological appearance, breast cancer can be classified as ductal or lobular carcinoma. Carcinoma in situ defines cancerous or precancerous cells growing within a particular tissue compartment without spreading to the surrounding tissue. The noninvasive forms of breast cancer with low grade are classified as Ductal Carcinoma in situ (DCIS) and Lobular Carcinoma in situ (LCIS). DCIS is the most common type of breast cancer with one case of DCIS detected per 1300 screening mammograms in North America and approximately 24% of all new cancers diagnosed in the United States are DCIS [12, 13]. Only about 10% of in situ breast cancers are lobular carcinoma in situ (LCIS).

Invasive Ductal Carcinoma (IDC) originates in the duct but it is not confined in the initial tissue. IDC can invade into the surrounding breast tissue, lymph nodes and spread throughout the body. Treatment of IDC varies depending on the size, grade and family history of breast cancer [12]. Surgery is typical nevertheless radiation therapy, chemotherapy, hormone therapy, and/or biologic therapy are usually combined with surgery [1].

From the view of pathology, breast cancer is described as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade). Normal mammary epithelial cells are commonly well differentiated and organized orderly to perform the specific function of the organ. Cancerous cells which

gain the uncontrolled cell division ability typically have irregular cell nuclei and disorganized shape. The less differentiated the cancer is, the worse prognosis it has.

Receptor status is an index to classify breast cancer from the molecular level. There are three main receptors which are also very important for the development of breast cancer used to define the breast cancer. According to the presence of the receptors on the surface or in the cytoplasm and nucleus of cell, breast cancer is divided as ER+ (estrogen receptor), PR+ (progesterone receptor), HER2+ and triple negative. The estrogen signaling blocker such as tamoxifen are often used to treat ER+ cancer cells for their dependency on estrogen for growth and the prognosis is good [13]. HER2+ cancer cell although is more aggressive but specific inhibitor or monoclonal antibody against HER2 activation increases the prognosis [14].

HER2

Epidermal growth factor receptor (EGFR) was first identified as potential oncogene in the early 1980s [15]. The mutation of the receptor was linked to the abnormal cell growth signals of cancer. Discovery of the other family members of EGFR led to identification of HER2/neu (human epidermal growth factor receptor 2, also known as c-neu or ErbB2). Its overexpression was found in breast cancer patients and associated with metastatic and aggressive forms of breast cancer [16]. EGFR family includes HER1 (Erb1, EGFR), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [17]. The structure of EGFR family members typically consists of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The exception is that HER3 does not have an active

intracellular tyrosine kinase domain. Ligand binding triggers receptor homodimerization or heterodimerization with another partner and activates tyrosine kinase domain via cross-phosphorylation (“autophosphorylation”) which then activates downstream effectors in a signaling cascade. The sites that are autophosphorylated and the downstream signaling molecules that are activated are determined by the identity of the ligand as well as by the dimeric partner [18]. HER2 does not have known ligand and it must form heterodimer with other members to be activated.

HER2 activation and HER2 signaling

Neu was first identified in about 30 years ago as an oncogene to induce neuroblastomas in rats treated with ethylnitrosourea [19, 20]. HER2 was then identified as the human version of neu from a cDNA library [21-23]. HER2 gene which encodes a 185-kd transmembrane growth factor receptor with tyrosine kinase activity is localized to chromosome 17q. HER2 is an orphan receptor with no known ligands identified. The structure of HER2 is in a fixed conformation which makes ligand binding impossible because its binding site is not accessible. However HER2 is a preferred partner of heterodimerization with other EGFR family members [24]. The fact lies on that the fixed conformation of HER2 resembles the ligand-activated state of the other members where it is in position to interact with the other receptors. Once another ligand-bound member dimerizes with it, HER2 activation occurs [25, 26].

Overexpression of HER2 can lead to HER2 spontaneous homodimerization and constitutive activation which promotes cell proliferation by activation of multiple signaling pathways. The main signaling pathways mediated by HER2 include the

phosphatidylinositol3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, signal transducer and activator of transcription protein 3 (STAT3), as well as Src tyrosine kinase and mammalian target of rapamycin pathway (mTOR) [17, 24, 27-29]. Ras/Raf/MAP-kinase and PI3-kinases/AKT are the predominately activated signaling upon oncogenic activation of HER2 and result in cell proliferation, cell survival, and malignant transformation [17]. (Fig. 1)

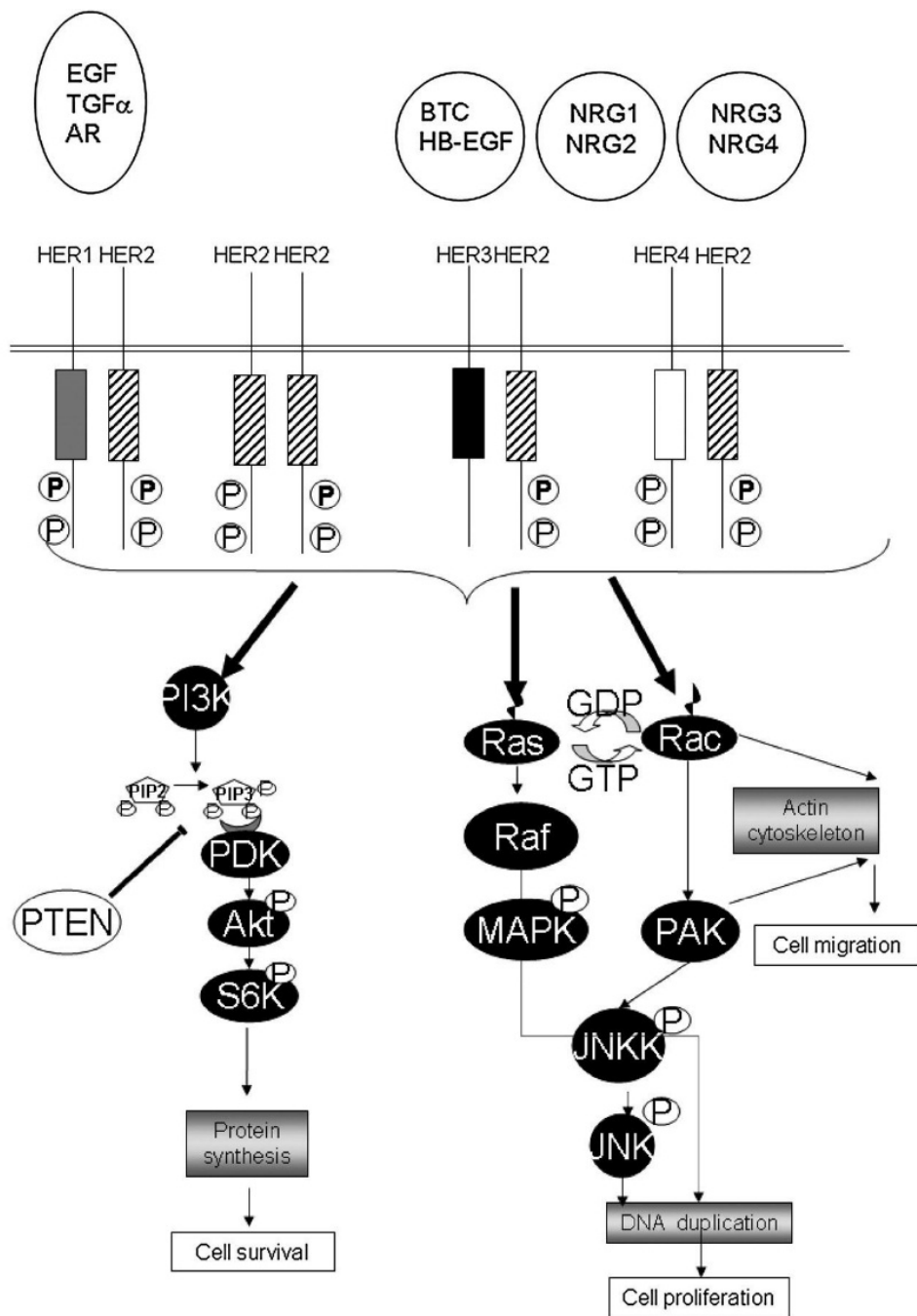


Figure1. Key pathways involved in downstream signalling after HER2 activation

HER2 in breast cancer

Clinical relevance of HER2 was first appreciated in breast cancer [16, 30]. The tumor cells of 25% to 30% of women with breast cancer overexpress HER2 receptor. In 85% of 120 publications (> 20,000 patients total), HER2 overexpression has been linked with adverse outcome in breast cancer, it is an indicator of aggressiveness and higher risk of recurrence and mortality (data from Genentech). NH2-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer. Agents targeting the HER2 pathway have been developed for use in the metastatic and adjuvant settings. Overexpression of HER2 leads to relative resistance to endocrine therapy. High level of HER2 also apparently causes lesser benefit from certain chemotherapeutic regimens in patients.

Anti-HER2 therapy

Trastuzumab (Herceptin) is a humanized monoclonal antibody directing against HER2 protein which is the first therapeutic antibody targeted to a specific oncoprotein to receive FDA approval. The mechanisms of trastuzumab action include inhibition of HER2 extracellular domain cleavage, acceleration of HER2 internalization and degradation, antibody- dependent cellular cytotoxicity(ADCC), inhibition of angiogenesis and inhibition on HER2 receptor signaling [31-36]. The studies suggested that transtuzumab inhibits cell growth through down-regulating PI3K activity and Akt function and up-regulating cyclin depend dent kinase (CDK) inhibitor P27^{kip1} expression which causes G1 phase cell cycle arrest [37].

Trastuzumab is used in clinics to treat patients with metastatic breast cancers that overexpress HER2 [38-41]. The response rate to trastuzumab as a single agent in patients who had not been previously treated is about 26% [42]. A combination of trastuzumab and chemotherapy treatments had the most effective results. 41.3% of women with HER2-positive tumors who had received both chemotherapy and trastuzumab have no evidence of any cancer remaining in the breast 15 months after the start of the trial, while only 19.5% in women who received chemotherapy alone had no cancer in the breast, the response rate increase by 50 percent. In addition, with combination therapy the median time of disease progression increase by 65 percent (7.4 vs 4.6 months) and the survival rate of patients after one year increased from 67 percent to 78 percent (25.1 vs 20.3 months) compared to with chemotherapy alone. Although, most of these cases did not cure the patient, combination treatments of trastuzumab and chemotherapy did prolong patient's lives by a significant amount. (Genentech, Inc. (produces Herceptin); MSNBC-Novel therapy targets cancer's roots; Breast Cancer Information)

However, the response rate to trastuzumab treatment is only 12% to 34% in women with HER2 gene-amplified metastatic breast cancers and many initial responders to trastuzumab relapse disease progression within a year[41-43] suggesting that there are both *de novo* and acquired therapeutic resistance. Multiple mechanisms of Herceptin resistance have been proposed. Firstly, HER2 proteolytic processing (HER2 shedding) produces a 95kDa truncated receptor p95 without extracellular domain (ECD) and releases a 105kDa soluble HER2 ECD. The retained HER2 p95 is unable to bind with trastuzumab but can form heterodimers with EGFR and HER3 or homodimers with itself, then activating downstream signaling, while the released circulating HER2 ECD compete binding with trastuzumab and abase its therapeutic

effects. Even if trastuzumab binds to the extracellular portion of full length HER2 sometimes it can not block HER2 dimerization and activation [44, 45] . Activation of the alternative signaling pathways due to PTEN deletion, PI3K mutations, autophosphorylation of MAPK and AKT as well as decrease of p27kip1 is another common resistant mechanism. In addition, overexpression of sialomucine MUC4 complex which disrupts interaction between HER2 and trastuzumab and increased signaling from other HER family members or other receptor families such as IGF1-IR and VEGFR all confer to transtuzumab resistance [46-51].

Another approach targeting HER2 is to directly block its intracellular tyrosine kinase activity. Lapatinib is a new generation of HER2-targeting drugs to overcome the limitations of treatment with trastuzumab. It is an orally active small-molecule which exerts tyrosine kinase inhibition on both EGFR and HER2 and classified as a dual tyrosine kinase inhibitor [52]. Lapatinib works intracellularly by reversibly binding to the cytoplasmic ATP-binding site at the tyrosine kinase (TK) domains of the EGFR or HER2 and blocking receptor phosphorylation and activation, thereby preventing subsequent downstream signaling events. In the clinic, lapatinib is active and improves survival in patients who has progressed after treatment with trastuzumab [43, 53]. Although lapatinib provides considerable clinical benefits in addition to trastuzumab, a large portion of HER2-positive tumors display resistance to these agents. Recent studies indicated that ER functions as a key escape pathway in ER-positive/HER2-positive cells treated with combination of lapatinib and transtuzumab. Therefore, blockade of the HER network along with ER inhibition may optimize the therapy in selected patients [54]. Therefore,

there is a need to develop new strategies to improve the efficiency of anti-HER2 therapy in breast cancer.

BRK

Breast tumor kinase (Brk), also called protein tyrosine kinase 6 (PTK6) is a ~ 56-60 kDa nonreceptor protein kinase and was cloned in 1994 in a screen for tyrosine kinases expressed in a metastatic breast tumor [55, 56]. Brk is highly expressed in approximately two thirds breast cancers while not expressed in normal mammary gland tissue [57], suggesting a tumor cell-specific function for the kinase. In addition to being detected in breast tumors, elevated expression of Brk has been detected in metastatic melanoma [58], colon tumors [59], T-cell lymphoma [60], and serous carcinoma of ovary [61]. In prostate cancers, although the expression of Brk is not significantly elevated, Brk translocates from the nucleus to the cytoplasm during tumor progression [62].

Structure of Brk

The structure of Brk is composed of Src-homology-2 (SH2) and SH3-like domains and a tyrosine kinase catalytic domain. Brk was originally classified as a Src-related kinase because its SH3, SH2, and kinase domains are arranged similarly to that of Src (SH3-SH2-Catalytic) [56]. However, Brk is only ~ 56% homologous to c-Src and it does not have characteristic myristoylation consensus sequences located at the N-terminal of Src. The myristoylation consensus sequences was regarded as be responsible for fatty acylation and membrane anchorage of Src family proteins[63], besides that SH2 and SH3 domains of Brk are not typical. So Brk is now considered to be a member of Frk family



Figure 2. Structure of Brk

which is a nonreceptor tyrosine kinase family different from Src family and the members include Frk, Brk, Srms and Sik [64]. An analysis of purified Brk protein using the Sf9/baculovirus system showed that Brk is regulated by autophosphorylation and by autoinhibition mechanisms [63]. Brk activity is increased by autophosphorylation at the tyrosine residue 342 of its activation loop within the kinase domain [63]. Its tyrosine residue 447 is analogous to the tyrosine residue 527 of Src, and mutation of tyrosine 447 (Y447) leads to constitutive activation of Brk due to SH2 domain accessibility [63]. Brk's SH3 domain plays a particularly important role in substrate recognition through governing phosphorylation of the substrates by Brk [65]. Experimental point mutation of the lysine residue 219 (K219M) completely abolishes the kinase activity of Brk [66]. (Fig. 2)

Known substrates and interacting proteins of Brk

Several Brk substrates have been identified and that may be categorized into three groups. The first group of substrates consists of the nuclear RNA-binding protein Sam 68 (Src-associated during mitosis, 68kDa) [67] and two Sam68-like proteins, SLM-1 and

SLM-2 [68]. Sam 68, an RNA-binding protein leading to growth inhibition, was first identified as a major target of Src during mitosis [69]. Like phosphorylation of Sam68 by Src, phosphorylation of Sam68 by Brk results in nuclear export of Sam68, inhibiting its RNA-binding function [67, 70]. The second group of substrates consists of the well-known signal transducer and activator of transcription(Stat) family, including Stat3 [71], Stat5b [72], and an adaptor-like Stat3 regulatory protein, BSK/STAP-2 (signal-transducing adaptor protein-2) [73]. BSK was recently shown to regulate the function of Stat3 and Stat5 [74, 75]. The suppressor of cytokine signaling 3 (SOCS3), a negative regulators discovered in cytokine signaling of the JAK-STAT pathway, binds with Brk and inhibits Brk activation of Stat3 [76]. The third group of substrates consists of GTPase activating proteins (GAPs) and related proteins including p190RhoGAP-A [77] and paxillin [78]. p190RhoGAP-A and paxillin are both involved in cell migration and cancer cell invasion [78].

Several additional proteins have been found to be associated with Brk, including HER1 (EGF receptor) [66, 79], HER2 [80], HER3 [81], Akt (when Brk is experimentally overexpressed) [82], IRS-4 [83], PSF [84], KAP3A [85]. These proteins may be substrates of Brk or their association with Brk may reflect Brk's adaptor protein function.

Brk and HER family in breast cancer

Brk-induced signaling events are implicated in cell proliferation, migration and invasion of breast cancer. More recently, Brk was shown to phosphorylate p190 RhoGAP

to regulate Rho and Ras and promote breast carcinoma growth, migration, and invasion [77].

Several pieces of evidence support that Brk has a particular interactive relationship with the HER family, which includes HER1/EGFR, HER2, HER3, and HER4. First, Brk interacts with EGFR. Brk potentiates EGF-induced proliferation of human mammary epithelial cells [66]. Our previous work indicated that Brk interacted with EGFR and sustained ligand-induced EGFR signaling. Upon activation by EGF, Brk directly phosphorylated EGFR on tyrosine 845 in the kinase domain, thus further potentiating EGFR kinase activity. In addition, Brk inhibits ligand-induced EGFR degradation through disassociating activated EGFR from casitas B-lineage lymphoma (cbl) which mediated EGFR ubiquitination. Experimental elevation of Brk sensitized breast cancer cells to cetuximab, an EGFR-blocking antibody, induced inhibition of cell signaling and proliferation.[79]. Experimental expression of Brk and EGFR in COS 1 cells can be associated upon EGF stimulation [82]. Chen *et al.* revealed that EGF induced Brk autophosphorylation which then induced paxillin phosphorylation and Rac activation to promote cellular migration. Tumor-promoting function of Brk is mediated in part by its phosphorylation of paxillin, which leads to activation of Rac1 via the adaptor protein Crk II [78]. Brk was recently shown to mediate EGF-induced and heregulin-induced activation of p38 mitogen-activated protein kinase(MAPK), which contributes in part to both proliferation and migration of breast cancer cells in response to these growth factors [86]. Other proteins that have been shown to be phosphorylated by Brk downstream of EGF are the RNA-binding proteins Sam68 and PSF [67, 84]. Upon EGF stimulation,

BRK phosphorylates PSF promoting its cytoplasmic localization and leading to cell cycle arrest.

Second, Brk enhances EGF-stimulated HER3 phosphorylation through increased recruitment of phosphatidylinositol 3-kinase to HER3, resulting in Akt activation [81], and regulates heregulin-induced activation of ERK5 and p38 MAPKs in breast cancer cells [86]. Third, Brk is simultaneously overexpressed with HER2 in tissue from invasive ductal breast carcinomas [87]. More recently, Brk has been shown to enhance the HER2-induced activation of Ras/MAPK signaling and cyclin E/cdk2 activity [80]. Brk cooperates with HER2 to increase the proliferative potential of HER2 positive tumors in a syngeneic mouse tumor model and to confer resistance to anti-HER2 therapy with lapatinib [80]. Together, all these findings suggest a clinically significant link between Brk and HER family members in tumorigenesis and development of resistance to HER family-targeted therapy.

CALPAIN-CALPASTATIN SYSTEM

Calpain was defined as Ca^{2+} dependent non-lysosomal cysteine proteases (proteolytic enzymes) and the first calpain (Calpain II) was discovered and purified by Dayton et al. in 1976 [88, 89]. The calpain-calpastatin system mainly composed of calpain-I, calpain-II and calpastatin is ubiquitously expressed in most tissues of vertebrates [90, 91]. Calpain-I and calpain-II differ primarily in their calcium requirements for proteolytic activity in vitro (calpain I requires micromolar Ca^{2+} levels and calpain II requires millimolar levels). Calpastatin, an endogenous proteinaceous

inhibitor of calpain, is a protein that specifically inhibits the proteolytic activity of calpain-I and calpain-II but of no other proteases known so far. Calpain-calpastatin system universally participate in a variety of cellular processes including cytoskeletal remodeling/cell mobility, cell signal transduction, cell cycle progression and apoptosis [90].

Properties of calpain

Both of calpain-I and calpain-II are heterodimers containing 28-kDa small subunit and an 80-kDa large subunit [90]. They have identical 28 kDa subunit and share 55– 65% sequence homology in the 80 kDa subunit which is slightly larger in calpain I than in calpain II (81889 Da vs 79900 Da). The amino acid sequences of calpains are highly conserved among many different species, from fungi to humans, with over 90% homology sequence among the mammalian [90]. The 80 kDa large subunit of calpain typically contains four structure domains among which domain I is cleaved after Ca^{2+} activation (autolysis) and domain II contains the active site with the catalytic triad of cysteine, asparagine, and histidine [92]. Besides the catalytic triad, domain II also can bind two atoms of Ca^{2+} and assist in calpain activation [93, 94]. Domain III containing two Ca^{2+} binding sites and a phospholipid-binding motif is responsible for substrate recognition and calpain activity regulation through specific electrostatic interactions [93, 95-97]. Moreover, domain IV contains the penta-EF hand, one of the most important features in calpain activation, which can bind Ca^{2+} , calpastatin, or the small subunit [90, 98]. The structure of 28 kDa small subunit of calpain only consists of two domains, V and VI. The function of domain V is to bind with the C-terminus region of domain IV in large subunits [99]. Domain VI, like domain IV in the large subunit, also has a penta-EF

hand which is for Ca^{2+} binding and heterodimer formation [100]. It seems that calpain recognizes its substrates not through the primary structure because calpain's substrates don't share the identical specific amino acid sequence. The tertiary structure rather than the primary amino acid sequences of the substrate are most likely responsible for directing cleavage by calpain. Calpain cleaves substrates at a hinge region between adjacent functional and regulatory domains, resulting in activation, inactivation, or destruction of the substrate proteins [89, 101].

Regulation of calpain activity

Calpain activity is sensitive to the intracellular $[\text{Ca}^{2+}]$ changes induced by extracellular stimuli under precise cell conditions [90, 94]. Depending on the condition of low or high $[\text{Ca}^{2+}]$ calpain demonstrate different conformational states [90, 94, 97, 102-107]. In resting conditions, calpain is located in the cytosol in an inactive form. Binding of Ca^{2+} to sites located in both the large and small subunits induces a conformational transition which align the essential residues located in the catalytic subdomains IIa and IIb and ultimately forms a functionally active catalytic site [94, 97, 104-107]. The conformational conversion was regarded as the limiting step in the calpain activation process which is followed by an autoproteolytic degradation that removes the N-terminal region of domains I and V and stabilizes the protease in a low Ca^{2+} requiring form. However, the precise events initiated by the binding of calcium and leading to the active state of calpain is still very unclear [108, 109].

The active enzyme form of calpain can also be regulated by calpastatin [110-112]. The conformational transition of calpain from the inactive state to active state is an

indispensable step for the calpain-calpastatin interaction [113-117]. Calpastatin must bind to calpain domain II and domain IV or VI to play inhibition on calpain activity. The association generally occurs within cells in the presence of physiological Ca^{2+} levels [108].

Properties of calpastatin

Calpastatin has eight splice variants mainly produced by alternative splicing of different exons in the N-terminal region of its transcript [118-120]. The structure of full length calpastatin contains one N-terminal region (L-domain) and four repetitive inhibitory units (domain I–IV) [119-127]. L-domain regulates L-type Ca^{2+} channels [128]; The other four domain I–IV are primarily responsible for calpain inhibition. Each unit of the other four repetitive inhibitory units is composed of three highly conserved regions called A, B, and C [129, 130] and possesses the potency to inhibit one calpain molecule respectively [122, 131]. Sequence B containing the calpain consensus sequence is necessary and sufficient for interacting and inhibiting calpain in vitro [130, 132, 133]. Sequences A and C was thought not essential for inhibition but can interact with domains IV and VI of calpain in the presence of Ca^{2+} [134-136].

Functions of calpain-calpastatin system

When a transient influx of calcium flows into the cell, it activates a small local population of calpains which catalyzes proteolysis of the target proteins and therefore alters the signal transduction pathways inside the cell. Under normal physiological conditions, calpain-calpastatin system plays important roles in regulating cell mobility,

cell cycle progression and apoptosis. Calpains participate in apoptotic cell death, and is an essential component of necrosis.

Calpain also has cell-type specific functions. In the brain, calpain-I exists in the cell body and dendrites of neurons and to a lesser extent in axons and glial cells, while calpain-II is found in glia and a small amount in axons [137]. They were shown to regulate long-term potentiation in neurons and play a role in memory. In addition, calpain participates in modulating skeletal muscle protein breakdown due to exercise and altered nutritional states [138]. Calpains is also involved in regulating clotting and the diameter of blood vessels as well as cell fusion in myoblasts.

The studies using genetically modified mice deficient in components of the calpain-calpastatin system revealed more vital functions of calpain-calpastatin system. Mice deficiency of *Capn1* gene which encodes both calpain-I and calpain II protein is embryonic lethality around embryonic day 10.5 (E10.5). The possible reason may be due to cardiac defects and hemorrhages [139]. Nevertheless, knockout of calpain I gene (*Capn1*) only did not result in visibly defects in fertility, development, or anatomy except platelet aggregation and integrin - 3 phosphorylation were somewhat restricted [140]. In contrast, knockout of calpain II gene (*Capn2*) caused mice to die as early as at E2.5 [141], suggesting that calpain II is more important than calpain I to embryonic development. Interestingly, calpastatin gene (*Cast*) knock out (KO) mice and transgenic (Tg) mice were essentially normal except the fact that *Cast* KO mice exhibited slightly abnormality in affective behavior. Calpastatin deficiency was reported to augment excitotoxic neurodegeneration [89, 142, 143]

The calpain-calpastatin system participates in a number of pathological mechanisms, including cataract, hypoxia, spinal cord injury, Alzheimer's disease, lissencephaly, muscular dystrophy, and ischemia. Deregulation of calpain activity following loss of Ca²⁺ homeostasis often lead to tissue damage resulting in myocardial infarcts, stroke, and brain trauma [144-146]. However its role involved in human cancer development was seldom studied.

EMT

Epithelial-mesenchymal transition (EMT) is a process whereby the cells lose epithelial cell polarity and cell-cell adhesion and gain a more or less fibroblastic phenotype with spindle-shape and migratory protrusions [147]. Acquisition of the ability to migrate and invade extracellular matrix (ECM) as a single cell is considered a functional hallmark of the EMT program. An orchestrated series of events happen during the process of EMT. Firstly, the cell-extracellular matrix(ECM) interactions are break to release epithelial cells from the surrounding tissue; secondly, the cytoskeleton is reorganized to allow free movement of the cell in three dimensions in the ECM; and finally a new transcriptional program is initiated to keep the cell mesenchymal phenotype [148]. Accompanying EMT, expression of epithelial markers such as E-cadherin, desmoplakin and cytokeratin decrease in abundance and the mesenchymal markers like vimentin, fibronectin and N-cadherin increase in abundance [149].

EMT in Physiological and Pathological Conditions

The conversion of epithelial cells to mesenchymal cells is a highly conserved cellular program. It is a central process for embryonic development. EMT has been

involved in many tissue remodeling events including neural crest development; mesoderm formation; secondary palate formation and heart valve development [150]. During embryogenesis, EMT program is not an irreversible process; the converted mesenchymal cells can revert to an epithelial cell state through a process named MET (mesenchymal-epithelium transition). The ability of a mesenchymal cell to revert to an epithelial cell indicates the cell plasticity and inter-conversion between epithelial and mesenchymal cells.

EMT also appears under other physiological and pathological conditions. In physiological response to injury, the keratinocytes at the border of the wound undergo EMT to acquire a metastable state which allows them to move and accelerates wound healing [151]. A similar process occurs in ovarian surface epithelium during the wound healing of post-ovulation [152]. EMT is involved in pathological organ fibrosis. Zeisberg reported that EMT contributes to liver fibrosis, cardiac fibrosis and renal fibrosis [153-155]. EMT of hepatocytes plays a role in the progression from cirrhotic livers to hepatocellular carcinoma in a mouse model [156]. TGF- β induces alveolar epithelial cell EMT during pulmonary fibrosis and renal fibrosis [157, 158], inhibition of TGF- β may be a reasonable strategy to revert the process of renal fibrosis [158-161].

Contribution of EMT to Malignant Progression

The majority of human solid tumors arise from various epithelial cell types. The epithelial cells are typically one sheet cell thick, tight connections between cells results in inhibition of movement away from the monolayers. In order for carcinoma cells to dissociate from neighboring cells to invade adjacent cell layers, they must lose cell-cell

adhesion and acquire motility which can be achieved through shape change. Therefore, EMT is considered playing a critical role in the early stage of cancer metastasis.

Although convincing evidence of EMT in clinical sample has not been observed which may due to the difficulties of distinguishing the mesenchymal cells derived from epithelial cell with the stromal cells and fibroblasts in the tumor tissues, EMT process has been recorded in multiple cancer cell models. EMT is detected in early lesion of HER-2 transgenic mice and in ductal carcinoma in situ in women [162]. At the invasion front of colorectal adenocarcinomas, individual tumor cells or small tumor cell aggregates become detached from the neoplastic glands. These cells have the morphological feature of mesenchymal cells and are regarded as closely associated with EMT [163]. In addition, EMT occurs at the invasion front of colon carcinoma and produces single migratory cells that lose E-cadherin expression and display β -catenin nuclear translocation, compared with the epithelial cells in central areas of the primary tumors [164]. Similar phenomenon appears in papillary thyroid carcinoma, breast carcinoma and cervical carcinoma.

Clinically, EMT protein expression has been shown to correlate with poor prognosis of non-small cell lung cancer, breast cancer, ovarian cancer and colon cancer.

Reprogramming of EMT in carcinoma endows incipient cancer cells with invasive and metastatic properties which facilitate cancer progression.

GENETICALLY MODIFIED MOUSE

A genetically modified mouse is a mouse with genome modified via using genetic engineering techniques. Genetically modified mice are commonly used as animal models

for studying human diseases that involve the overexpression or misexpression of a particular protein. Two basic technical approaches are used to produce genetically modified mice. The first is transgenic mouse which is created through directly microinjection of cloned DNA into a single stem cell of the mouse embryo. As a result, the transgene randomly and stably integrates into the mouse genome and exists in all cell types and at any time in the developing embryo and postnatal animal [165]. Transgenic mouse model is universally used for the purpose of inserting new genetic information into the mouse genome to examine the gain function of the exogenous genes. The other method involves modifying embryonic stem cell gene through homologous recombination [166]. Embryonic stem cells from blastocyst of mouse are isolated and transfected with modified DNA sequences homologous to the target gene in tissue culture. The embryonic stem cells with homologous recombination is selected and then injected into a new blastocyst of mouse to form a chimeric blastocyst. Chimeric blastocyst implanted into a pseudo-pregnant mouse would eventually develop chimeric mice which need to mate with wild type mice to generate heterozygous mice. Normally to get a mouse with both alleles containing homologous recombination gene, two or more generations of selective breeding is needed. Homologous recombination method is often used to manipulate a single gene especially knocking out the target gene. Knock-out mice with a particular gene on both alleles being replaced with an inactive gene to determine the function of the particular gene by observing the phenotype of the knock-out mice.

(<http://www.bio.davidson.edu/Courses/genomics/method/homolrecomb.html>)

Transgenic mouse models

The first transgenic mouse was created by Rudolf Jaenisch in 1974. He inserted a DNA virus into an early-stage mouse embryo and found that the inserted genes were present in every cell; although the transgenes could not pass to the offspring [167]. In 1981, people injected purified DNA instead into a single-cell mouse embryo and successfully created the transgenic mice which could deliver the transgenes to subsequent generations [168, 169] .

Inducible transgenic mouse models

Inducible transgenic mouse models allow for the control of gene activation in a cell type specific and time specific manner which is suitable for physiologic analysis or mimicking disease states. An effective inducible transgenic mouse model is characterized by low background levels of the transgene and but the expression of the transgene can be induced to high levels with sub-physiological levels of inducing agents. The dose of the agent administered also can regulate the expression levels of the transgene.

The tet-operon/repressor bi-transgenic system and the estrogen receptor (ER) - ligand binding domain are two most commonly used methods to control gene expression in mouse models. Other methods less commonly used include the progesterone receptor (PR) - ligand binding domain and the lac and GAL4 inducible systems.

The tetracycline-dependent regulatory (tet) system are typically designed for a precise spatial and temporal control of transgene expression [170]. The tetracycline controlled transactivator responsive minimal Tet promoter tet-O, also called tetracycline-

responsive element (TRE or tet-operator), relies on two components: a transgene that allows the expression of the tetracycline transactivator (tTA) or the reverse tetracycline transactivator (rtTA), and a transgene that contains a gene of interest (target transgene) under the control of a synthetic tTA/rtTA dependent promoter tet-O. In the absence of Dox, tTA binds to the tet-O promoter and activates the transcription of the target transgene. In the presence of Dox, the conformation of tTA has been changed which results in tTA come off the tet-O-promoter stopping the transcription of the target transgene (Tet-off). Tet-off system provides the possibility to express a gene in a cell-specific manner and stop its expression by Dox administration. On the contrary, rtTA, a complementary genetic module, is unable to activate the tet-O promoter alone. Only in the presence of Dox, rtTA binds and activates tet-O promoter and initiates the transcription of the target transgene (Tet-on). Tet-on system is uniquely suitable for rapid gene activation by addition of Dox [171]. In both Tet-Off and Tet-On system, tTA and rtTA expression can be driven by tissue-specific promoters, thereby regulating target gene expression in a tissue-specific manner. These two systems have been successfully employed in transgenic mice for studying various biological functions [172, 173]. (Fig. 3)

The ER ligand binding domain can be used with transcription factors, kinases and most commonly fused with Cre recombinase. The Cre recombinase from bacteriophage P1 is a necessary tool for conditional gene activation and inactivation in the genetically modified mouse for the study of gene functions [174]. Cre recombinase inactivates gene expression by directly excising the target gene, while it activates gene expression through removing stop cassettes from the transgenes allowing the expression of target gene. A fusion protein composed of Cre recombinase and ER ligand binding domain makes the

induction of recombinase activity possible in specific cells at defined time points in which the Cre activity can be induced efficiently by the addition of tamoxifen (Extrogen) [175, 176].

Figure 3. The TET System (http://jaxmice.jax.org/news/2011/Alzheimer_Leukemia.html, thank Jackson Laboratory for the approval of the use of the diagram)

A) In the Tet-Off system, transactivator protein (tTA) binds to the tetracycline-responsive promoter element (TRE) and activates transgene expression. In the presence of tetracycline (Tc) or doxycycline (Dox), tTA is unbound and transgene expression is off.

B) In the Tet-On system, reverse tetracycline-controlled transactivator protein (rtTA) is unbound in the absence of Dox, resulting in an inactive transgene. In the presence of Dox, rtTA binds to the TRE and activates transgene expression.

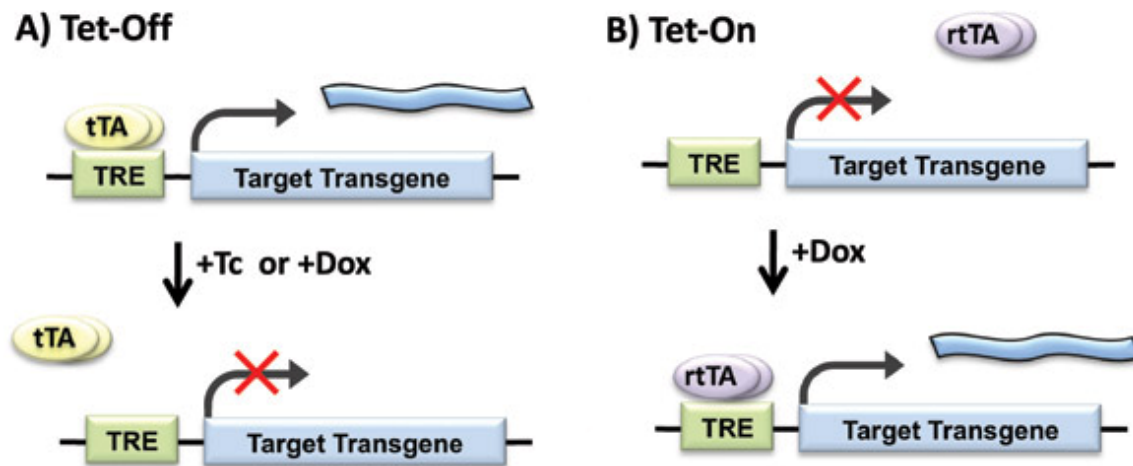


Figure 3. The TET System

(The Jackson Laboratory : JAX® Mice harboring Tet Expression Systems)

Mammary gland development

Mammary gland development mainly occurring postnatally is characterized by a highly dynamic and hormonally-driven process. After birth, the mammary ducts elongate into the mammary fat pad. From the age around four weeks to the age about 7–8 weeks, the mammary ducts grow rapidly with the ducts invading towards the lymph node. At this time, terminal end buds (TEBs) which is a highly proliferative structure emerge at the tips of the invading ducts and expand significantly. The invading ducts soon reach the end of the mammary fat pad by the end of pre-pubertal time. During the puberty, terminal end buds shrink in size and become less proliferative. Under the hormonal stimulation, secondary branching develop from the primary ducts and fill all mammary fat pads eventually establishing an arboreal network of hollow ducts emanating from the nipple. Ductal development is slowed down by sexual maturity to undergo estrous cycles (pro-estrous, estrous, met-estrous, and di-estrous). During estrous cycle, the mammary gland experiences dynamic changes between cells proliferate and regression in an ordered manner due to cycling hormonal change [177]. Upon pregnancy, the ductal systems undergo a marked increase of cell proliferation and differentiate alveolar structures in the ductal branches to prepare the gland for lactogenesis. Milk is produced by the luminal cells in the alveoli. At the time of weaning when suckling stimulus of the pups is removed, involution of the mammary gland is initiated accompanying lactation stops. Post-lactational involution of the mammary glands involves the controlled apoptosis of mammary alveolar epithelial cells, remodeling of the extracellular matrix (ECM) and restoring of the stromal adipocyte compartment which revert the gland to a near virgin

state. The regression process in mouse model usually takes about 10 days to complete [178, 179].

Breast cancer transgenic mouse models

The milestone of transgenic mouse model in breast cancer research was the experiments conducted by Philip Leder and coworkers in 1984 [180]. They created the transgenic mice carrying the hybrid gene which was fused the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV) to the human c-myc proto-oncogene. These transgenic mice eventually developed breast tumor by expressing the human myc protein in their mammary glands [180] .

Generally, the tumorigenic phenotype of breast cancer is defined by the transgene, while the cell type affected and the temporal onset of the phenotype are determined by promoters controlling transgene expression. In addition to the LTR of MMTV, promoters from milk protein genes have been used to control transgene expression too in breast cancer, this category includes promoters of whey acidic protein (WAP), beta lactoglobulin (BLG) and β -casein. Expression of these control elements is targeted to the mammary epithelium and stimulated by lactogenic hormones. So the tumor latency in multiparous mice is shorter than that in virgin mice. It was reported that promoter of C3(1), a subunit of the rat prostate steroid binding protein (PSBP), successfully targeted the expression of the SV40 large T-antigen (Tag) to the epithelium of both the mammary and prostate glands and induced mammary and prostate cancers [181]. MMTV-LTR and C (3)1 promoter driven transgene is preferentially activated in ductal and alveolar cells, and transgene controlled by promoters from milk protein genes commonly is expressed

only in alveolar cells. Moreover, high activity of MMTV-controlled transgenes can be detected earlier than WAP-driven genes [182] (Table1).

Table 1
Mammary gland specific promoters

Promoter	Origin	Expression	Activation	References
MMTV-LTR	Mouse mammary tumor virus	Breast epithelial cells, several other tissues	Steroid hormones	[183]
WAP	Whey acidic protein	Secretory mammary epithelium	Lactogenic hormones	[184, 185]
C3 (1)	Rat prostate steroid-binding protein (PSBP)	Epithelial cells of prostate and mammary gland	Estrogen (ductal and alveolar mammary epithelium)	[181]
B-LG	Bovine β - lactoglobulin	Mammary gland	Pregnancy and lactation	[186, 187]
MT	Metallothionein	Most mammary cells	Zn 2+	[188]

In general, oncoproteins and growth and cell-cycle regulators can induce mammary tumors in transgenic mice. The table 2 summarizes the known oncogenes which expression in mammary tissue can initiate breast carcinogenesis in transgenic mice.

Table 2

Transgenic (TG) mouse models of breast cancer

TG mouse model	Expression	Tumor incidence (%)	Tumor latency (months)	Metastasis incidence (%)	References
Single-transgenic mice					
MMTV-Cox2	Mammary gland	>85	7	N/A	[189]
MMTV-Wnt1	Mammary gland	60	8		[190-192]
MMTV-Neu	Mammary gland	100	6.8	72	[191, 193]
MMTV-Neu activated	Mammary gland	100	3	20	[183, 194]
MMTV-Neu (YB)	Mammary gland	100	6	65	[194, 195]
MMTV-Neu (YD)	Mammary gland	100	3.6	44	
MMTV-PyMT	Mammary gland	100	1–6	>85	[191, 196, 197]
MTB-TAN	Mammary gland	100	N/A	92	[191, 198]
	Mammary gland	N/A	10	N/A	[191, 199]
MT-Met					
C3(1)-Tag	Mammary gland	100	3–6	N/A	[191, 200]
	Mammary gland	100	6.2	high	[201]
Wap-Notch4					
	Mammary gland	12–83	11	N/A	[202]
Wap-T-NP					
	Mammary gland	100	6	14	[191, 203]
Wap-Ras					
Wap-HGF	Mammary gland	89	1–2	22	[204]
	Mammary gland	50–100	>9	38	[191, 205]
H19-IGF2					
Composite-transgenic mice					
	Mammary gland deletion	100	10–18	50	[206]
p53fp/fp MMTV-Cre Wap-Cre					
p53 +/- MMTV- Δ N- β -catenin	Mammary gland	100	9.8	39	[207]
CD44 -/- MMTV-PyMT	Mammary gland	100	3.5	66	[208]

MMTV-Neu;SR2F	Mammary gland	86.8	8	12	[209]
MMTV-NeuYB;T β RI(AAD)	Mammary gland	100	8.9	65	[195]
MMTV-NeuYD;T β RI(AAD)	Mammary gland	100	4.4	44	
MMTV-NeuYB;T β RII(Δ Cyt)	Mammary gland	100	6	65	
MMTV-rtTA/TetOp-TGF- β 1S223/225; MMTV- PyMT	Mammary gland		1.8	>10-fold	[68]
MMTV-Neu; S100A4	Mammary gland		12	50	[16,113]
MMTV-Wnt1; int2	Mammary gland	90	4		[7,77]
MMTV-PyMT; uPA $-/-$	Mammary gland	100	1.5	31	[45,51,52]
MMTV-PyMT; Plg $-/-$	Mammary gland	100	1.5	25	
MMTV-PyMT; VEGF	Mammary gland	100	1–2	100	[91]
MMTV-PyMT; MEKK1 $-$ $-/-$	Mammary gland	100	3	25	[53]

Because tumor progression is a multistep process involving different signaling pathways, transgenic mice carrying more than one transgene have been generated. A typical example was the pioneering study conducted by Leder and his coworkers in 1987. They crossed MMTV/v-Ha-ras transgenic mice with MMTV/c-myc transgenic mice to

yield hybrid mice which were co-expressed MMTV/v-Ha-ras and MMTV/c-myc genes and showed a dramatic and synergistic acceleration of tumor formation [210].

Approximately 50% of primary human breast cancers harbor p53 mutation, however, absence of one or two p53 alleles alone did not lead to the formation of mammary tumors in transgenic mouse model. Deletion of functional p53 was observed to accelerate mammary tumors in context of the wnt1 transgenic mice. Deletion of Brca1 gene in both alleles in mammary tissue resulted in breast tumors after a latent period of approximately 1 year [211], while concomitant deletion of one allele of p53 accelerated tumor formation. The generation of bitransgenic mice help identify the parallel and interconnected signaling pathways in breast cancer development [212] .

HER2/neu transgenic mouse

HER2 is overexpressed in 20–30% of primary breast cancers which is correlated with poor prognosis of patients. The involvement of HER2 in the initiation and progression of breast cancer is further supported by analysis of HER2 transgenic mouse. In 1988, the first transgenic mice of neu (the rat homolog of HER2) is generated by targeting overexpression of activated neu, a mutated form of neu with valine instead of glutamic acid at residue 664 in the transmembrane domain, to the mouse mammary gland under the control of the murine mammary tumor virus (MMTV) promoter. Consequently these mice developed multifocal mammary tumors as early as 11–13 weeks [183, 213, 214]. Later in 1992, transgenic mice with overexpression of wild type neu under the MMTV promoter were reported developing multifocal mammary tumors after a latency period of 17–48 weeks. Breast tumors also arose in the transgenic mice generated with

overexpression of wild type human HER2 under MMTV although with a longer latency of about 28.6 weeks [215]. However, wild type human HER2 under the whey acidic protein(WAP) promoter in the transgenic mice did not induced mammary neoplastic transformation [216].

CHAPTER 2: HER2 INCREASES BRK PROTEIN STABILITY

INTRODUCTION

HER2, a member of the epidermal growth factor receptor (EGFR) family which is called class I receptor tyrosine kinases [24, 217], has been found amplified or overexpressed in 30% of human breast cancers [30] and also in many other cancer types [218]. Patients with HER2-overexpressing breast tumors have higher incidences of metastasis, lower survival rates and shorter times to relapse than patients without the overexpression.

Breast tumor kinase (Brk) is a nonreceptor protein tyrosine kinase that is highly expressed in approximately two thirds of all breast cancers [57, 86] . Brk was first cloned from a human metastatic breast tumor and was originally classified as a Src-related kinase because it is 46% homologous to c-Src and composed of SH3, SH2, and kinase domains arranged in a way similar to those of Src (SH3-SH2-Catalytic) [55, 56, 219]. However, Brk does not possess N-terminal myristoylation consensus sequences of Src and its SH2 and SH3 domains are atypical [63]. Thus Brk is distinguished from Src family kinases and belongs to nonreceptor tyrosine kinase family known as the Frk family that includes Frk, Brk, Srm, and Sik [64]. Compared to Src, much less is known about the biological functions of Brk in human cancer, but several Brk substrates and interacting proteins have been identified. Brk is functionally related to the human epidermal growth factor (EGF) receptor (HER) family, which includes HER1/EGFR, HER2, HER3, and HER4. More recently, Brk was shown to be simultaneously overexpressed with HER2 in tissues from patients with invasive ductal breast carcinomas

[80, 87] and to enhance the HER2-induced activation of Ras/MAPK signaling and cyclin E/cdk2 [80]. In a syngeneic mouse model, Brk was shown to cooperate with HER2 to increase the proliferative potential of HER2-positive tumors and to confer resistance to lapatinib, aHER2/EGFR dual inhibitor [80]. However, the mechanisms of co-overexpression of Brk and HER2 and the interaction between these two molecules in breast cancer are largely unknown.

In this part, we studied the mechanism underlying the frequent co-expression of Brk and HER2 in breast cancer. We first demonstrated a correlation between the protein levels of Brk and HER2 in a panel of breast cancer cell lines and then studied the regulatory effects of HER2 on Brk levels by either experimental elevation of HER2 or knockdown of HER2 by RNA interference. Our data suggest that Brk is more stable in HER2-overexpressing cells than in corresponding cells expressing a low level of HER2. Further mechanistic studies showed that Brk is degraded through a calpain-mediated proteolytic pathway, which is inhibited by HER2. HER2 inhibits calpain activity through up-regulating calpain endogenous inhibitor, calpastatin, expression which requires MAPK activation. Brk plays important roles in mediating the functions of HER2 and silencing Brk sensitize the cell to lapatinib treatment. Our findings provide novel mechanistic insights into the interaction between Brk and HER2 and justify targeting Brk as a novel strategy for enhancing HER2-targeted therapies.

MATERIALS AND METHODS [220]

Reagents

Antibodies directed against total Akt, serine 473 (S473)-phosphorylated Akt, threonine 202/tyrosine 204 (T202/Y204)-phosphorylated extracellular signal-regulated kinase (Erk), tyrosine 877 (Y877)-phosphorylated HER2, total Stat3, tyrosine 705 (Y705) phosphorylated Stat3, total Src, tyrosine 416 (Y416) phosphorylated Src and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology, Inc.

Antibodies against total Erk, Brk, calpastatin and calpain I were purchased from Santa Cruz Biotechnology, Inc. Antibodies against HER2 and small molecular N-acetyl-leucyl-leucyl-norleucinal (ALLN), calpain substrate II, MG132, PD98059 and LY294002 were purchased from Calbiochem/EMD Chemicals, Inc. All other chemicals were purchased from Sigma-Aldrich Corp. The constitutively active MEK1 (S217E/S221D) pcDNA3.1 construct was subcloned from pMCL vector containing the MEK1 mutant insert that was originally provided by Dr. Natalie Ahn (University of Colorado). Transfection of these constructs was done with Lipofectamine 2000 (Invitrogen).

Breast cancer cell lines and culture

The breast cancer cell lines MCF7, T47D, ZR75B, MDA157, MDA361, SUM190, MCF-10A, HCC1954, MDA453, MDA435, MDA468, MDA231, SKBR3, and BT474 were originally purchased from American Type Culture Collection. The breast cancer cell lines SUM102 and SUM149 were obtained from Dr. Steven Ethier's laboratory (The University of Michigan and the Karmanos Cancer Institute). The BT20 and HS578T cell lines were obtained from Dr. Mien-Chie Hung's laboratory (MD Anderson Cancer

Center). MCF7-HER2 cells were created by transfection of MCF7 cells with a wild-type HER2 construct, as described previously [221]. Except for SUM102 cells, which were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum (FBS), 5 µg/mL insulin, 1 µg/mL hydrocortisone, 2.5 µg/mL fungizone, and 5 µg/mL gentamicin, all cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin and cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Western blot analysis

Cells were lysed in a lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 25 µg/mL leupeptin, and 25 µg/mL aprotinin and clarified by centrifugation (14,000g for 30 min at 4°C). The protein concentration of the cell lysates was determined using the Bradford Coomassie blue method (Pierce Chemical Corp.). For immunoprecipitation studies, cell lysates were incubated with primary antibodies, and the resultant immune complexes were precipitated with protein A-Sepharose beads (GE Healthcare). Whole-cell lysates or immunoprecipitated proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred onto nitrocellulose by Western blotting, and probed with various primary antibodies and horseradish peroxidase-labeled secondary antibodies. The signals were visualized with an enhanced chemiluminescence detection kit (GE Healthcare).

Calpain *in vitro* proteolytic assay and enzymatic activity assay

The calpain *in vitro* proteolytic assay was performed as described in the literature [222]. The cell lysates (50 µg protein per sample) were prepared in the lysis buffer, as described in the preceding paragraph, and were incubated with varying amounts of purified calpain I (Sigma-Aldrich Corp) in 50 µL of calpain reaction buffer (50 mmol/L Tris-HCl, pH 7.4, 10 mmol/L CaCl₂, 30 mmol/L NaCl, 5 mmol/L 2-mercapto-ethanol) for 90 min at 37°C. Proteolytically cleaved Brk protein was examined by Western blotting.

Calpain enzymatic activity was measured as described in the literature [223]. Lysates were prepared in a calcium-free imidazole buffer (63.2 mmol/L imidazole-HCl, pH 7.3, 10 mmol/L 2-mercapto-ethanol, 1mmol/L EDTA, and 10mmol/L EGTA) plus 10 mmol/L digitonin. Equal amounts of cell lysates (500 µg protein) and fluorescently labeled calpain substrate II (Calbiochem, EMD) (25 µmol/L) were incubated in the calcium-free imidazole buffer or in the buffer containing calcium (63.2 mmol/L imidazole-HCl, pH 7.3, 10 mmol/L 2-mercapto-ethanol, and 10 mmol/L CaCl₂) at 37°C for 30 min in triplicate sets. Fluorescence generated after the reaction was measured with a fluorometer (FLUOstar Omega, BMG LABTECH) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Calpain activity, expressed as relative fluorescent units (RFU) per milligram of cell lysate protein, is the difference between the RFU of calpain substrate II cleaved by the calpain in the lysates in the presence and absence of calcium.

SiRNA/shRNA transfection

HER2 small interfering RNA (siRNA) oligonucleotides (#1 sense sequence: GGGAAACCUGGAACUCACC; complement sequence: GGUGAGUUCCAGGUUUGCCC; #2 sense sequence: GGACAUCUUCCACAAGAAC; complement sequence: GUUCUUGUGGAAGAUGUCC) were ordered from Ambion/Applied Biosystems. HER2 lentiviral short hairpin RNA (shRNA) constructs (HER2 shRNA #1: target DNA sequence: GCCTTCGACAACCTCTATTAC, HER2 shRNA #2: target DNA sequence: TGTCAGTATCCAGGCTTTGTA, HER2 shRNA #3: target DNA sequence: GAGATCACAGGTTACCTATAC) and Brk lentiviral shRNA constructs (Brk shRNA #1: target DNA sequence: ACCTCTCCCATGACCACAATA, Brk shRNA #2: target DNA sequence: TACCTCTCCCATGACCACAAT, Brk shRNA #3: target DNA sequence: GTGCAGGAAAGGTTCAACAAT) were purchased from Sigma-Aldrich Corp. Brk siRNA oligonucleotides (#1 sense sequence: AAGGUGAUUUCUCGAGACAAC; complement sequence: GUUGUCUCGAGAAAUCACCUU; #2 sense sequence: UCUUGAGAGCUUGGCCUUAUU; complement sequence: UAAGGCCAAGCUCUCAAGAUU) were purchased from Dharmacon/Thermo Fisher Scientific. Transfection of siRNA oligonucleotides was performed in a six-well plate (1×10^5 cells/per well) with Lipofectamine 2000 (Invitrogen, Inc.), using the methods recommended in the manuals provided by the manufacturer. Knockdown of targeted gene expression was examined 48 h after siRNA transfection by Western blotting with specific antibodies. HER2 and Brk shRNA constructs were co-transfected with a lentivirus packaging plasmid into HEK 293T cells. The resulting lentiviral particles were used to

infect the human breast cancer cell line, 24 hours post-infection, 2 ug/ml of puromycin was added to select for infected cells for a week. Cells were harvested. Knockdown of HER2 and Brk expression level were detected by Western blotting. β -Actin expression was used as an internal control.

Cell growth and survival assay

Cells were cultured in 24-well plates with 0.5 ml medium per well at 37°C in a CO₂ incubator. After transient transfection of the cells with Brk siRNA or control siRNA for 48 h, the cells were incubated for an additional 2 h after addition of 50 μ L/well of 10 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were then lysed in a lysis buffer (500 μ L/well) containing 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) at 37°C for at least 6 h. The relative number of surviving cells in each group was determined by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value in each treatment group was then normalized to that of untreated cells as a percentage of the OD value of the control cells and plotted against the treatments.

RESULTS

HER2 overexpression in breast cancer cells up-regulates Brk protein expression

Western blot analysis of Brk and HER2 expression in a panel of 17 breast cancer cell lines and MCF-10A shows that Brk is frequently expressed in two thirds of these cells and HER2 is expressed in one thirds of these cells (Fig. 4A). To determine whether there is correlation between the expression of HER2 and Brk, we quantified the western blot band of Fig. 4A using ImageJ densitometry software; the column graph represented the relative expression level of Brk and HER2 to internal control β -actin in 17 breast cancer cell lines and MCF-10A. A scatter plot of relative Brk expression versus HER2 expression was generated, and the correlation analyses were performed by Spearman's method (Fig. 4B). The Spearman correlation coefficient was 0.6182 with a P value <0.01, suggesting that there is a significant correlation between the expression of HER2 and Brk.

To determine the regulation between Brk and HER2, we treated HER2- and Brk-co-overexpressing breast cancer cell lines SUM190 with two HER2-specific siRNA and two Brk specific siRNA. Knockdown of HER2 expression in SUM190 resulted in marked decrease in the Brk protein level which is correlated to the reduction of HER2 expression level, whereas knock down of Brk expression did not change the HER2 expression level, suggesting that HER2 can regulate Brk expression (Fig. 4C). In addition, MCF7-HER2 cells, which express a high level of HER2 through stable transfection, contained a higher level of Brk than did the parental MCF7 cells (Fig. 4D), providing further evidence that the Brk level is regulated by HER2.

Figure 4. Correlation of Brk and HER2 protein expressions in breast cancer cell lines

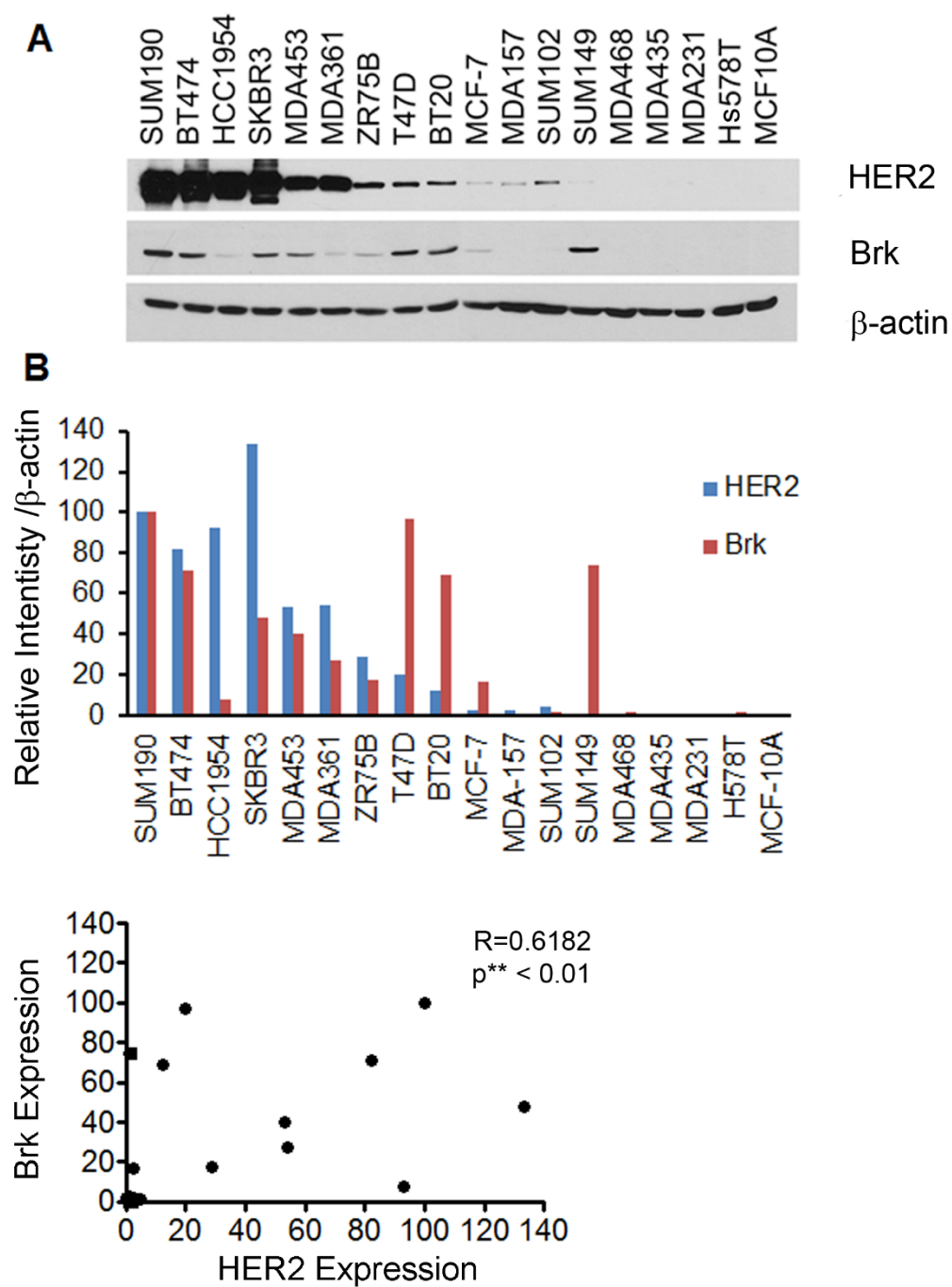
A). A panel of 17 breast cancer cell lines and MCF10A nonmalignant mammary epithelial cells were analyzed by Western blot analysis with antibodies directed against Brk and HER2. β -actin served as a protein loading reference.

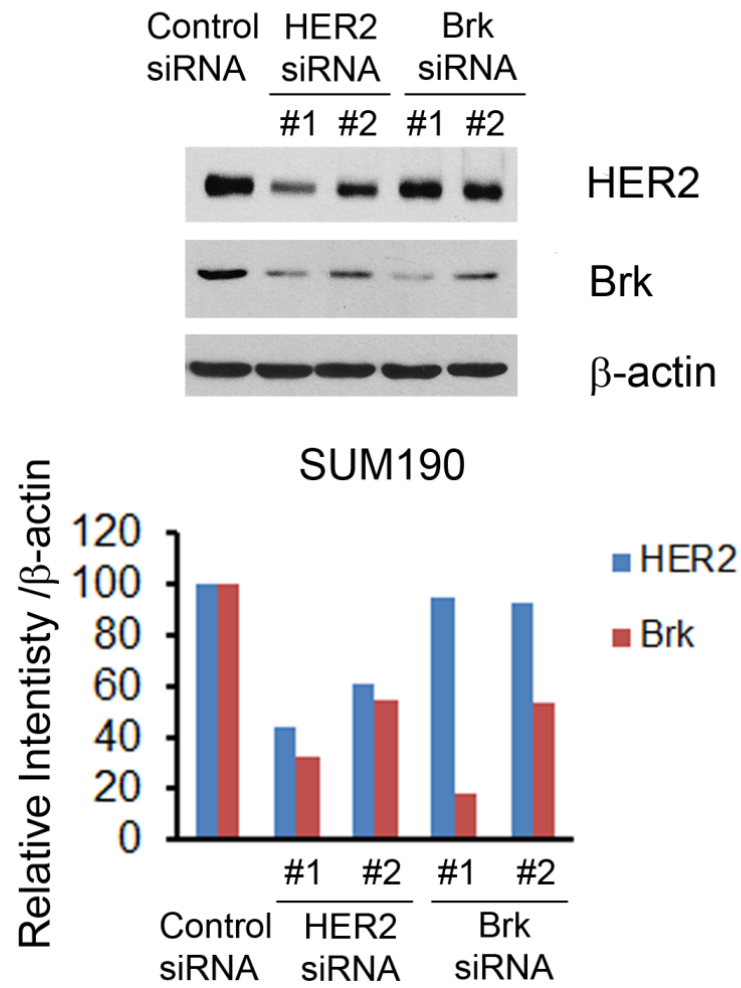
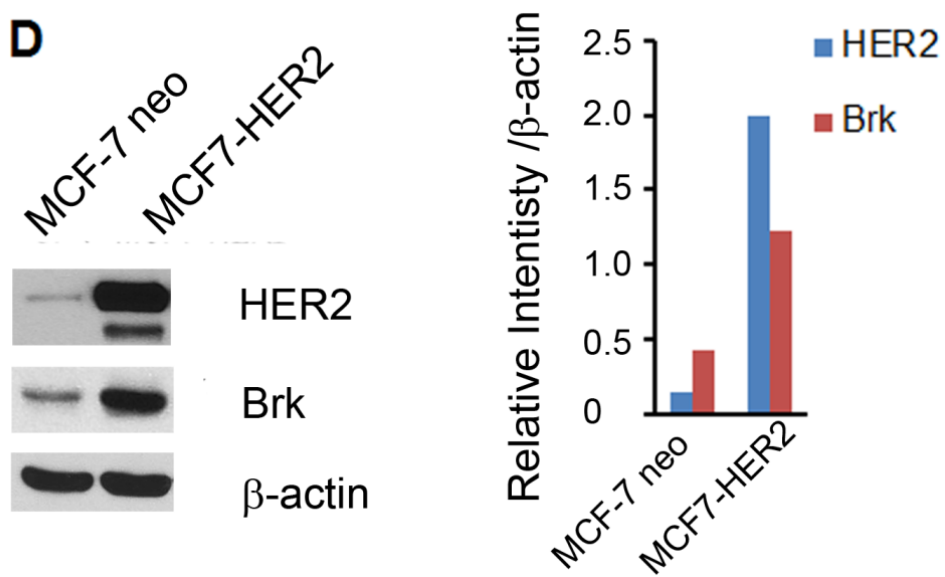
B). Western blot results in the cell lines of panel A were quantified by Image J densitometry software and relative expression of Brk and HER2 to internal control β -actin were represented by the histogram. Spearman analysis was used to analyze the correlation between Brk and HER2 expression in these cell lines.

C). SUM190 cells were transiently transfected with two different HER2-specific siRNA and two different Brk-specific siRNA respectively for 48 h. The cells were then lysed and analyzed by Western blotting with indicated antibodies. The result of western blot was quantified by Image J densitometry software and relative expression of Brk and HER2 to internal control β -actin were represented by the histogram.

D). MCF-7 was stably transfected with HER2 construct to get the MCF7-HER2 cell, both MCF-7 and MCF7-HER2 were lysed and analyzed by Western blotting with indicated antibodies. The result of western blot was quantified by Image J densitometry software and relative expression of Brk and HER2 to internal control β -actin were represented by the histogram.

Figure 4



C**D**

HER2 up-regulates Brk by increasing Brk protein stability

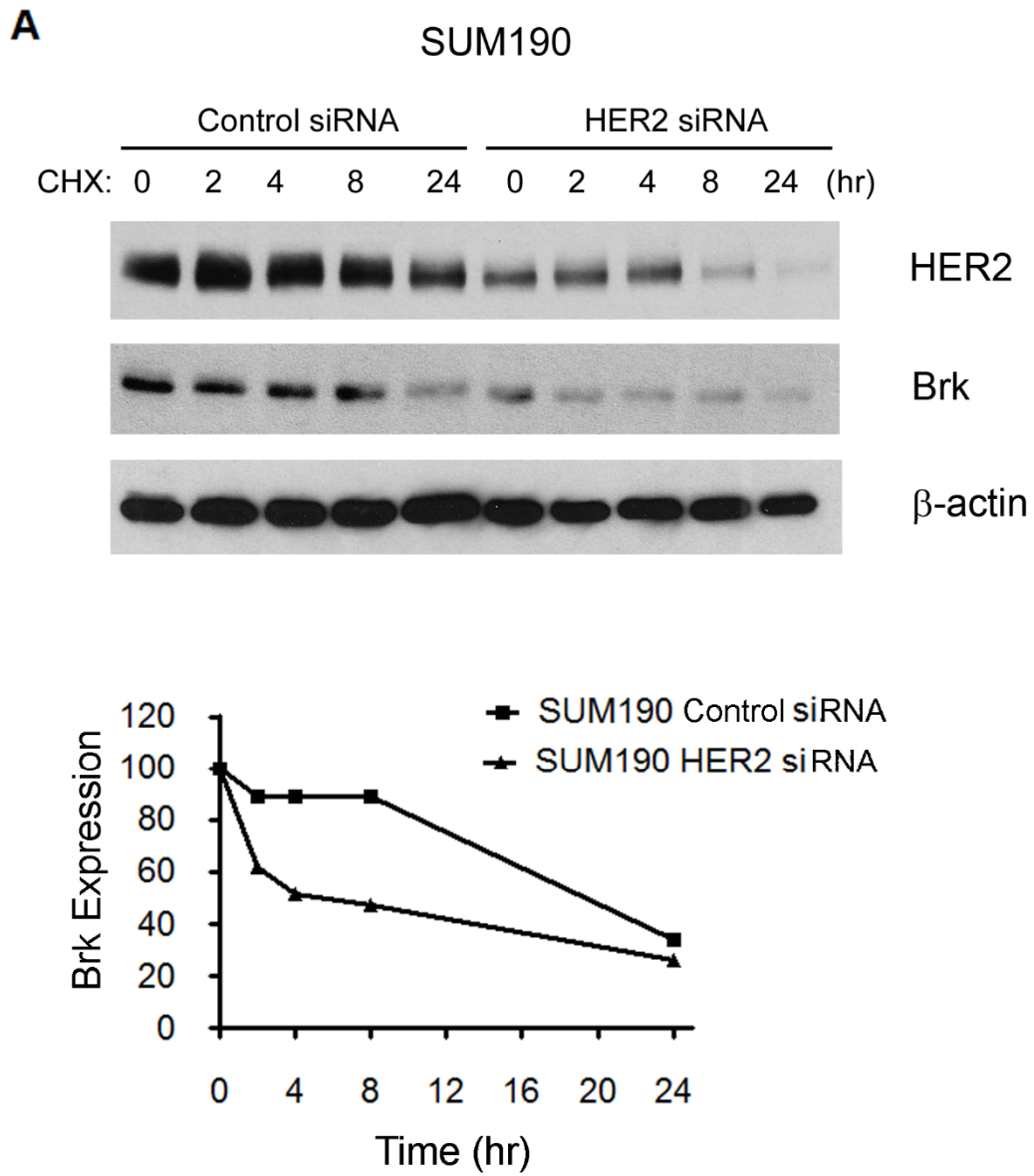
To test whether HER2 influence Brk protein stability, we compared Brk protein stability in SUM190 with or without HER2 silencing. Consistent with the finding shown in Figure 4C, the expression of Brk was decreased in SUM190 cells after HER2 silencing and the protein was further reduced as early as 2 h treatment of cycloheximide (CHX), which inhibited synthesis of new proteins. By contrast, the Brk protein level in SUM190 with control siRNA had no change until 24 h treatment of CHX (Fig. 5A). We also compared Brk protein stability in MCF7neo and MCF7-HER2. The basal level of Brk was higher in the MCF7-HER2 cells than in the MCF7neo cells. A decrease in the Brk protein level was seen in the MCF7neo cells as early as 2 h after exposure of the cells to CHX. In the MCF7-HER2 cells, the decrease in the Brk protein level was not seen until sometime between 4 and 24 h after CHX treatment (Fig.5B). These findings indicate that Brk is more stable in HER2 overexpression cells, suggesting that overexpression of HER2 can enhance the stability of Brk.

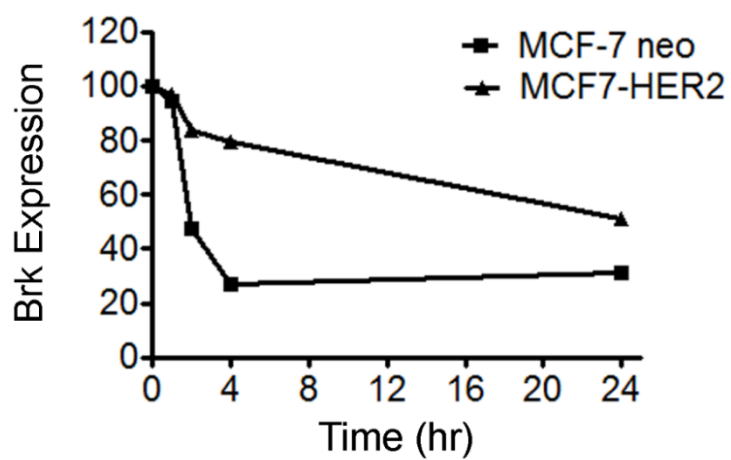
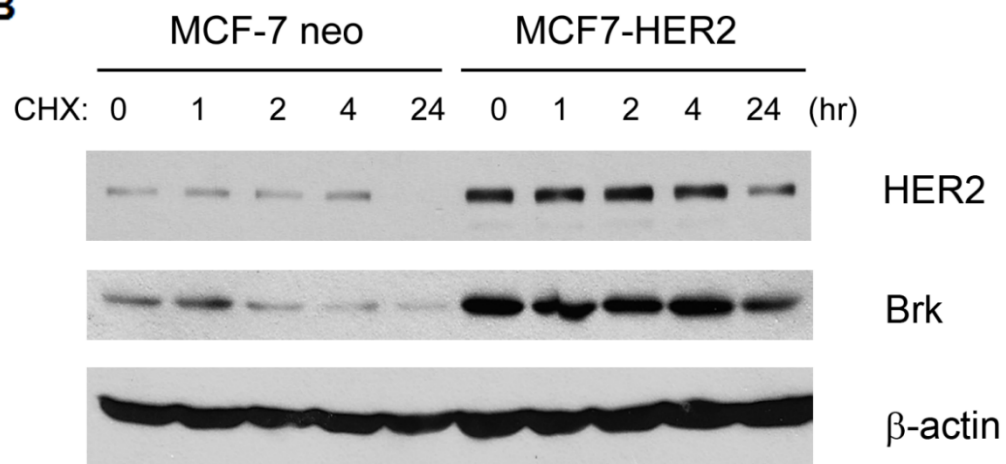
Figure 5. Overexpression of HER2 enhances Brk protein stability

A). HER2 silencing in SUM190 decrease Brk protein stability. SUM190 were transiently transfected with HER2-specific siRNA or control siRNA for 48 h and then were exposed to 50 $\mu\text{g/mL}$ cycloheximide (CHX) in 0.5% FBS medium for the times indicated. The level of Brk and HER2 was detected by Western blotting. β -actin was used as a loading control. The western blot result of Brk and β -actin were quantified by Image J densitometry software and relative expression of Brk to internal control β -actin were described as the linear graph.

B). Brk is more stable in MCF7-HER2 cells than in MCF7neo cells. MCF7neo and MCF7-HER2 cells were exposed to 50 $\mu\text{g/mL}$ cycloheximide (CHX) in 0.5% FBS medium for the times indicated. The level of Brk was detected by Western blotting. β -actin was used as a loading control. The western blot result of Brk and β -actin were quantified by Image J densitometry software and relative expression of Brk to internal control β -actin were described as the linear graph.

Figure 5



B

HER2 up-regulates Brk by inhibiting its calpain mediated proteolysis

Recently, Kang et al reported that Brk was degraded through proteasomal pathway in breast cancer cells [224]. Our study demonstrated that both proteasomal inhibitor MG132 and calpain inhibitor ALLN were capable of restoring Brk expression after application of CHX for 24 hours in both SUM190 and MCF7-HER2 (Fig. 6A), suggesting that besides through ubiquitin-proteasome pathway, Brk also was degraded through calpain pathway, a non-lysosomal and calcium-dependent cysteine protease pathway known to regulate Src kinase activity by cleaving Src [225, 226]. To further validate whether Brk is a substrate of calpain, we examined the sensitivity of Brk to protein cleavage by calpain I. We first incubated MCF7-HER2 cell lysates with calpain I purified from human plasma. Similar to Src, Brk was cleaved by calpain I *in vitro* (Fig. 6B), indicating that Brk is subject to calpain-mediated proteolytic cleavage. Because overexpression of HER2 can enhance the stability of Brk (Fig. 5A and B), we then determined whether HER2 has any effect on inhibiting calpain expression and/or activity, which might inhibit the degradation of Brk. We measured the calpain activity in the lysates of MCF7 cells, with and without HER2 overexpression, and in the lysates of SUM190 cells, with and without HER2 knockdown. Overexpression of HER2 in MCF7 cells or knockdown of HER2 in SUM190 cells with siRNA had no effects on the level of calpain expression in these cells (Fig. 6C upper); however, the enzymatic activity of calpain was significantly lower in MCF7-HER2 cells than in MCF7-neo cells. Similarly, the enzymatic activity of calpain was significantly higher in SUM190 cells after knockdown of HER2 expression. During these assays, the cell lysates treated with a calpain substrate mimetic, N-acetyl-leucyl-leucyl-norleucinal (ALLN), that functions as a

calpain inhibitor [225], served as negative controls for the calpain activity assay. And the correlation between calpain activity represented by relative fluorescent units (RFU) and protein dosage of both MCF-7 and SUM190 cells was detected to validate the effectiveness of calpain assay. (Fig. 6C lower). Overall, this inverse relationship between the levels of HER2 expression and calpain activity suggests that overexpression of HER2 leads to inhibition of calpain activity, which accordingly inhibits Brk degradation and leads to co-overexpression of Brk and HER2.

To further provide *in vivo* evidence demonstrating that Brk is degraded through a calpain dependent proteolytic pathway and that the proteolytic pathway is inhibited by HER2, we examined the levels of Brk in two paired breast cancer cell lines expressing high and low levels of HER2, treated with and without ALLN. Consistent with the findings shown in Figure 5C, the basal level of Brk was higher in MCF7-HER2 cells than in MCF7neo cells (Fig. 6D, lanes 1-6 versus lanes 7-12), and the degradation rate of Brk was faster in MCF7neo cells than in MCF7-HER2 cells (Fig. 6D, lanes 1-3 versus lanes 7-9). In addition, we found that Brk degradation was inhibited by ALLN in both MCF7neo and MCF7-HER2 cells (Fig. 6D, lanes 1-3 versus lanes 4-6 and lanes 7-9 versus lanes 10-12. In contrast, the degradation of HER2, which is known to occur mainly through ubiquitination and the proteasomal pathway, was not affected by the treatment of cells with ALLN (Fig. 6D, lanes 7-9 versus lanes 10-12).

To explore whether the same is true in breast cancer cells with high endogenous levels of HER2 and Brk, we performed the reverse experiment to determine the impact of HER2 expression on the Brk protein level and stability by knocking down HER2 expression in SUM190 cells. Treatment of SUM190 cells with a HER2-specific siRNA

successfully reduced the expression of HER2 in the cells (Fig. 6E). Consistent with the findings shown in Figures 4C and 5A, the Brk level was markedly reduced in the SUM190 cells after HER2 knockdown, compared with the high basal level of Brk in the control siRNA-treated cells (Fig. 6E, lanes 7-12 versus lanes 1-6). In addition, consistent with the findings from MCF7neo and MCF7-HER2 cells, degradation of Brk is dependent on calpain mediated protein cleavage in both control siRNA and HER2 siRNA treated SUM190 cells (Fig. 6E, lanes 1-3 versus lanes 4-6 and lanes 7-9 versus lanes 10-12). Knockdown of HER2 expression in SUM190 cells led to a substantial decrease in the Brk level 4 h after CHX treatment; in contrast, the level of Brk, although it was relatively lower owing to knockdown of HER2 expression, remained stable up to 24 h after CHX treatment in the presence of ALLN (Fig. 6E, lane 9 versus lane 12). Together, these findings provides important *in vivo* evidence that Brk is degraded by calpain mediated protein cleavage, and calpain mediated Brk proteolysis is less active in HER2-overexpressing cells than in cells expressing a low level of HER2.

Figure 6. Brk is degraded through a calpain-mediated proteolytic cleavage pathway which is inhibited by HER2

A). Proteasome inhibitor and calpain inhibitor rescued Brk from degradation caused by protein synthesis inhibition. SUM190 and MCF7-HER2 cells were treated with CHX (50 $\mu\text{g}/\text{mL}$) combined with or without proteasome inhibitor MG132(5 μM) or calpain inhibitor ALLN (20 μM) for 24 hours in 0.5% FBS DMEM .Lysates were analyzed by Western blotting for Brk and β -actin.

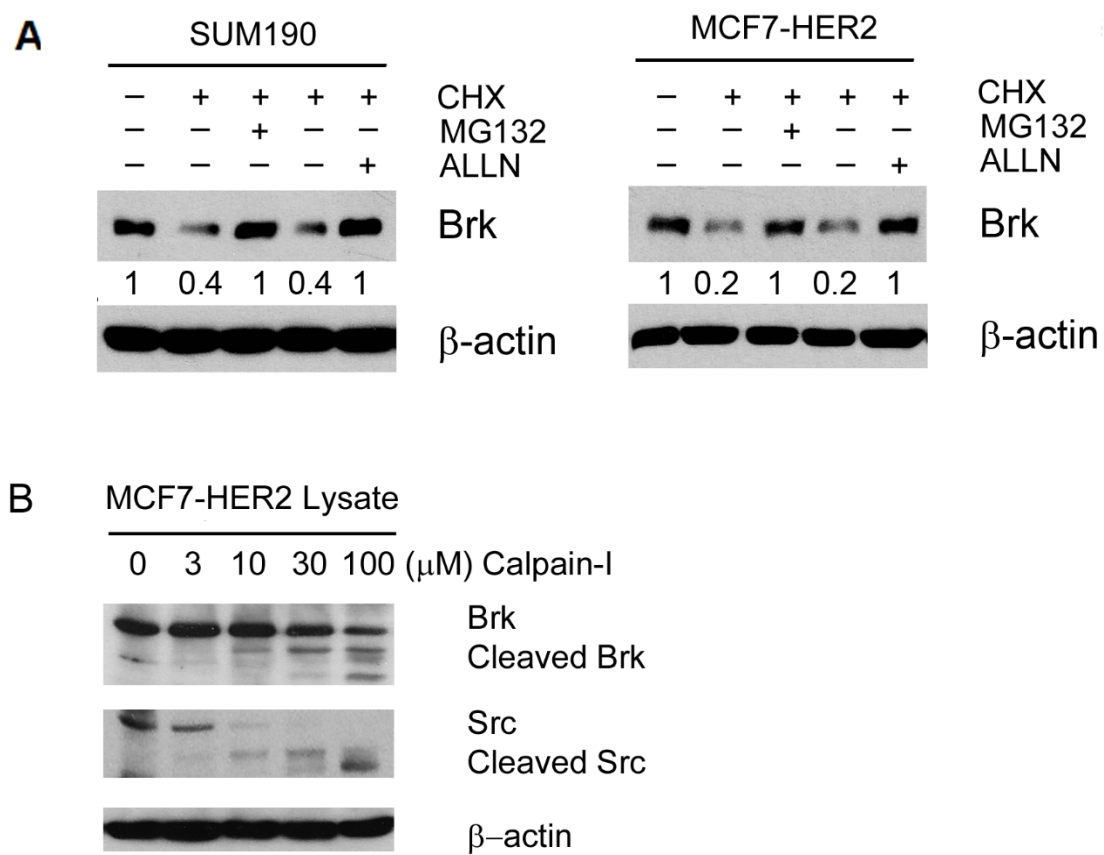
B). Proteolytic cleavage of Brk in vitro by calpain I. MCF7-HER2 cell lysates were incubated with increasing doses of purified calpain I in a reaction buffer for 90 min at 37°C. The calpain-cleaved products were analyzed by Western blotting with antibodies against Brk, Src and β -actin.

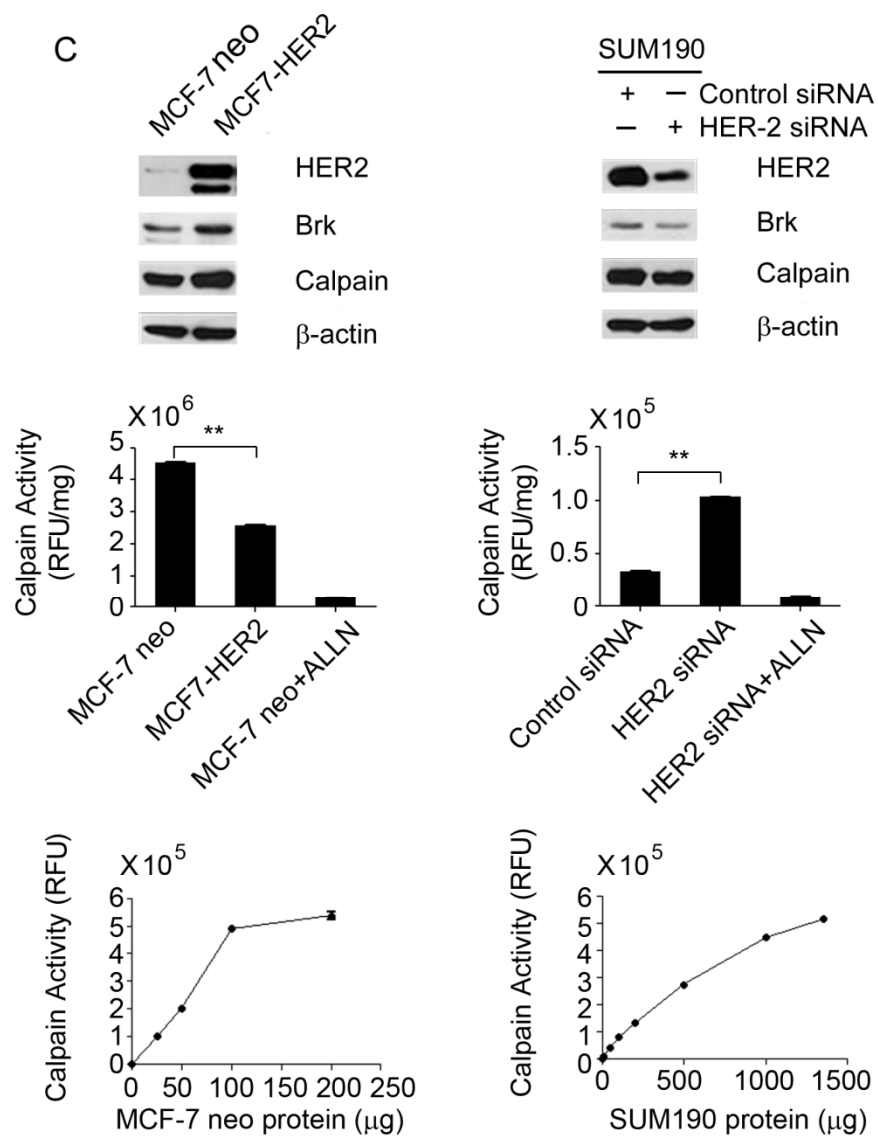
C). Inverse relationship between HER2 expression and calpain I activity. Equal amounts of MCF7neo and MCF7-HER2 cell lysates, and equal amounts of control siRNA-treated SUM190 cell lysates and HER2 siRNA-treated SUM190 cell lysates (each in triplicate) were incubated with the fluorescently labeled calpain substrate II at 37°C for 30 min. Calpain activity was determined as described in Materials and Methods. Lysates of MCF7neo and HER2 siRNA-treated SUM190 cells with addition of 25 μM ALLN were used as negative controls of calpain activity in vitro. The same lysates were also analyzed by western blotting for Brk, HER2, calpain and β -actin. Different protein dosages of MCF-7 and SUM190 lysate were performed calpain assay to validate the effectiveness of calpain assay. ** $p < 0.01$

D). Increase in Brk level after HER2 overexpression and dependence of Brk degradation on calpain. MCF7neo and MCF7-HER2 cells were treated with CHX (50 $\mu\text{g}/\text{mL}$), with or without ALLN (10 μM), for 4 h and 24 h. Lysates of untreated and treated cells were analyzed by Western blotting for Brk and HER2. β -actin served as a loading control. The bar graph showed the relative expression level of Brk/ β -actin quantified by Image J densitometry software.

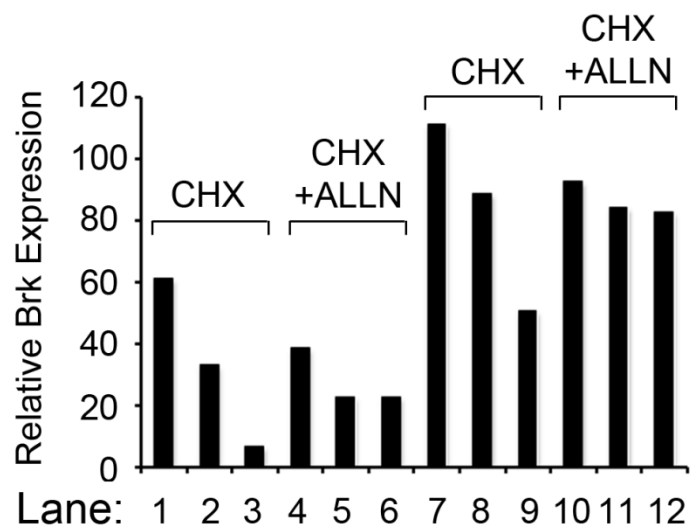
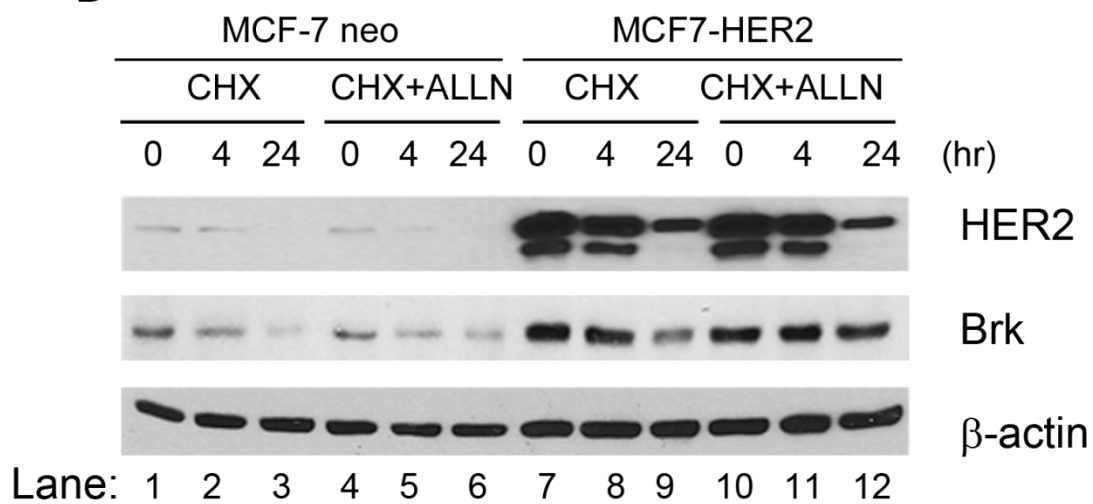
E). A decrease in Brk level after knockdown of HER2 expression and dependence of Brk degradation on calpain. SUM190 cells were transiently transfected with HER2 or control siRNA for 48 h. The cells were then treated with CHX (50 $\mu\text{g}/\text{mL}$), with or without ALLN (10 μM), for 4 h and 24 h. Lysates of untreated and treated cells were analyzed by Western blotting for Brk and HER2 levels. β -actin served as a loading control. The bar graph showed the relative expression level of Brk/ β -actin quantified by Image J densitometry software.

Figure 6



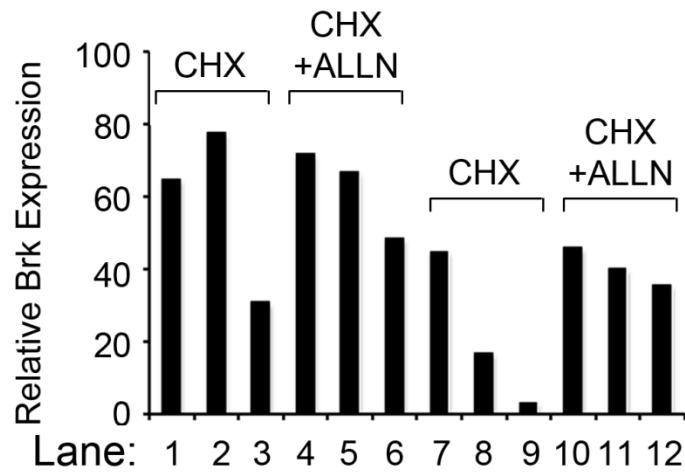
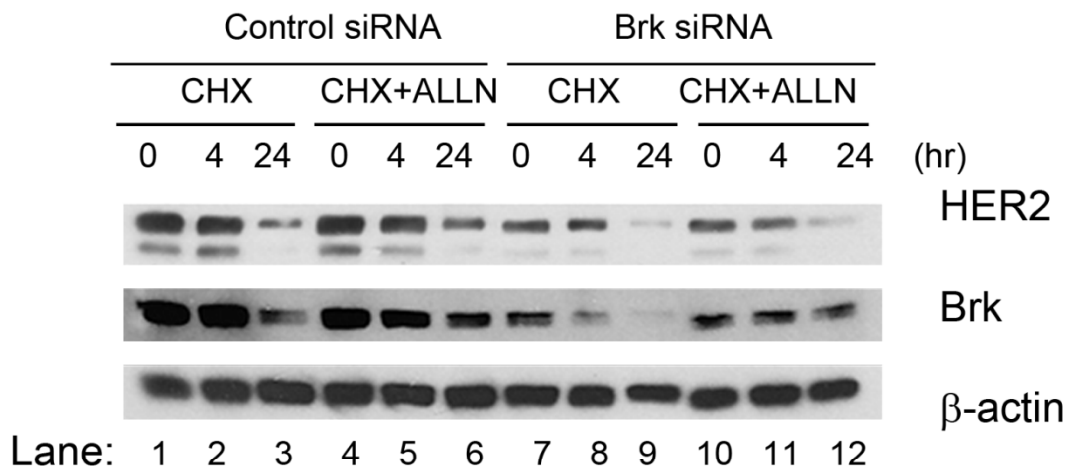


D



E

SUM190



HER2 inhibits calpain activity through up-regulating calpastatin protein expression

Calpastatin is an endogenous calpain specific inhibitor. The activity of calpain is strictly regulated by in vivo calcium concentrations and interactions with calpastatin. To examine whether HER2 modulates calpain activity through changing the expression level of calpastatin, we knockdown HER2 expression via lentiviral shRNA infection of SUM190, the reduction of HER2 expression through three different HER2 shRNA resulted in relevant down-regulation of calpastatin expression as well as Brk expression, and the decrease of HER2 expression caused corresponding elevation of calpain activity (Fig. 7A).

Moreover, the calpastatin expression in MCF7-HER2 was significantly higher than that in parental MCF-7 cells (Fig. 7B). Besides Brk expression increase, Src, another calpain substrate, expression increased too in MCF7-HER2 cells. And HER2 silencing in SUM190 was followed by the expression decrease of Brk, Src and calpastatin (Fig. 7C). The results indicated that HER2 up-regulated calpastatin expression to achieve the inhibition on calpain activity.

Figure 7. HER2 inhibits calpain activity through up-regulating calpastatin, expression

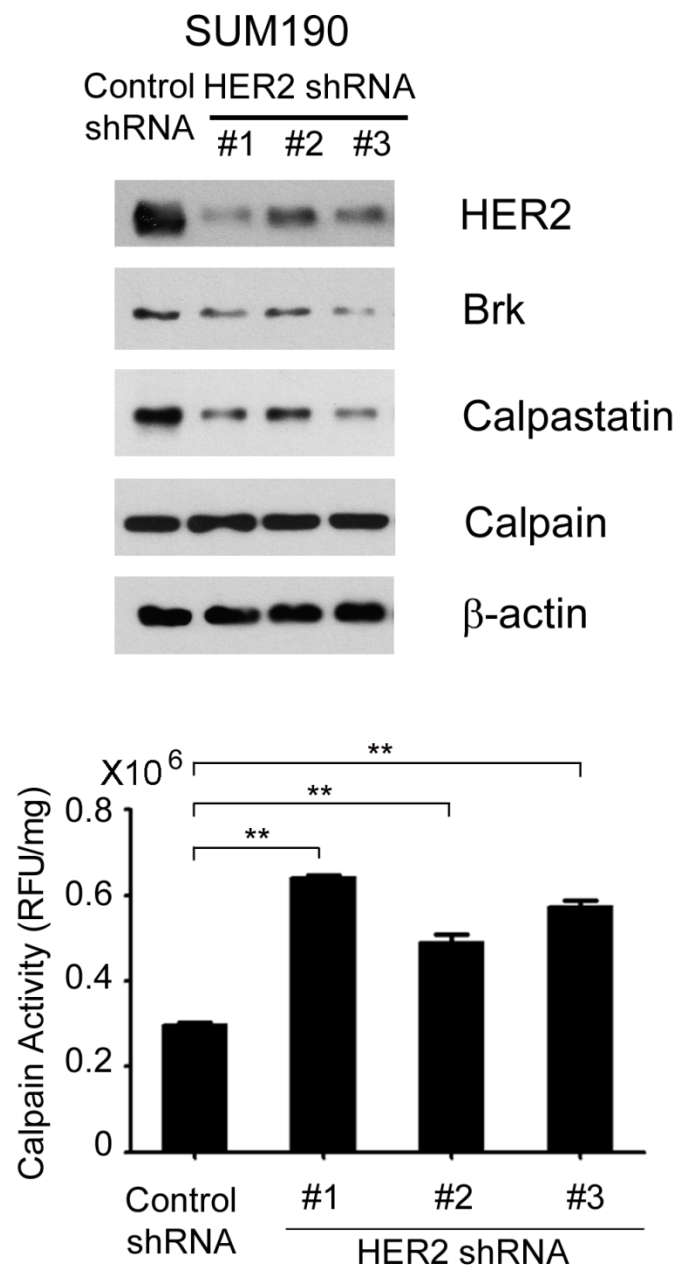
A). Knockdown of HER2 expression down-regulated calpastatin expression. SUM190 was infected with three different lentiviral shRNA against HER2. 48 hours later, the stable cells were selected with 2ug/ml puromycin for a week, Lysates of cells with control shRNA and HER2 shRNA were analyzed by Western blotting for indicated antibodies. β -actin served as a loading control. The cells also were lysated upon calpain assay as described in materials and methods. ** $p < 0.01$

B). MCF-7 and MCF7-HER2 cells were lysed and analyzed by western blotting with indicated antibodies.

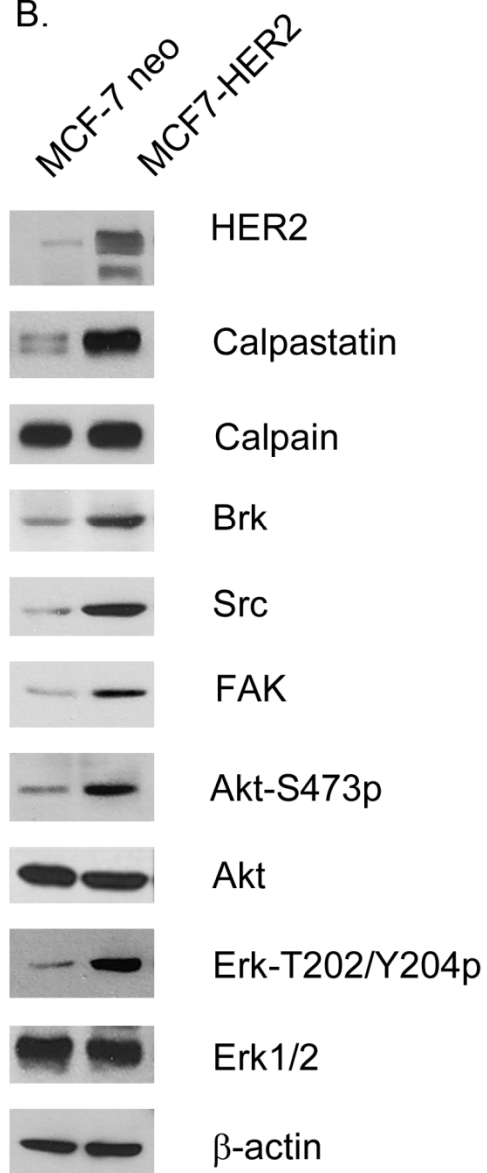
C). SUM190 were transiently transfected with HER2-specific siRNA or control siRNA for 48 h , and the cells were lysed and analyzed by western blotting with indicated antibodies.

Figure 7

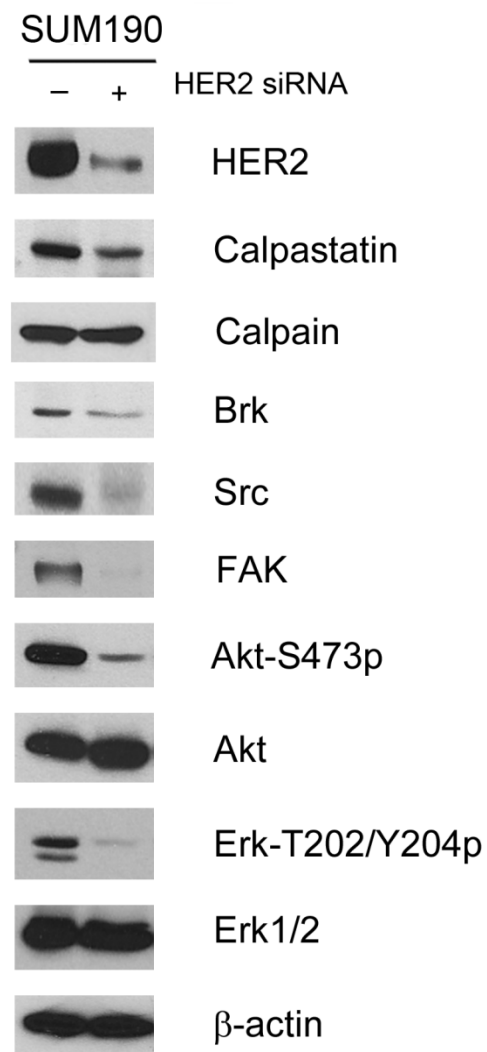
A.



B.



C.



HER2 enhances calpastatin protein expression through MAPK pathway

To date, the mechanism how calpastatin expression is regulated remains unclear. To determine which downstream signal pathways of HER2 was involved in the regulation of calpastatin expression by HER2. We treated MCF7neo and MCF7-HER2 with PI3K inhibitor, LY294002 which blocks Akt activation, and MEK1 inhibitor PD98059 which inhibits MAPK activation. The results proved that LY294002 successfully abated the phosphorylation of Akt and decreased calpain activation in both MCF-7 and MCF7-HER2 cells but it exerted no inhibition on Brk and calpastatin expression (Figure 8A). In contrast, MEK1 inhibitor PD98059 which down-regulates MAPK phosphorylation but not Akt phosphorylation led to relevant reduction of Brk expression and calpastatin expression. In the parallel calpain assay, PD98059 treatment elevated calpain activity in a dosage dependent manner in MCF7-HER2 although showed slight inhibition on calpain activity in parental MCF-7 cells (Figure 8B). We further confirmed the observation in SUM190 with PD98059. Dephosphorylation of MAPK resulted in marked Brk and calpastatin down-regulation as well as calpain activity enhancement (Figure 8C). To provide direct evidence to prove that MAPK is implicated in regulation of calpain activity by HER2, SUM190 was co-transfected with HER2 siRNA and with or without constitutively active (CA) MEK1, an upper activating kinase of MAPK, construct. Expression of MEK1 (CA) restored MAPK phosphorylation in the presence of HER2 siRNA and partially increased calpastatin and Brk expression. Simultaneously, expression of MEK1 (CA) prevented calpain activation caused by HER2 silencing (Figure 8D). We got the same result in BT474, another Brk and HER2 co-overexpression breast cancer cell line (Figure 9). Together, these data suggest that HER2

through activating MAPK pathway enhances calpastatin expression which then inactivate calpain enzyme, and eventually lead to Brk expression increase.

Figure 8. HER2 up-regulates calpastatin expression through MAPK pathway instead of Akt pathway

A). HER2 up-regulates calpastatin expression not through Akt pathway. MCF-7 and MCF7-HER2 cells were treated with indicated dose of Akt inhibitor, LY294002, for 24 hours in 0.5%FBS DMEM. Cell lysates were prepared and analyzed by Western blotting with indicated antibodies. The cells with the same treatment were underwent calpain assay. * $p < 0.05$; ** $p < 0.01$

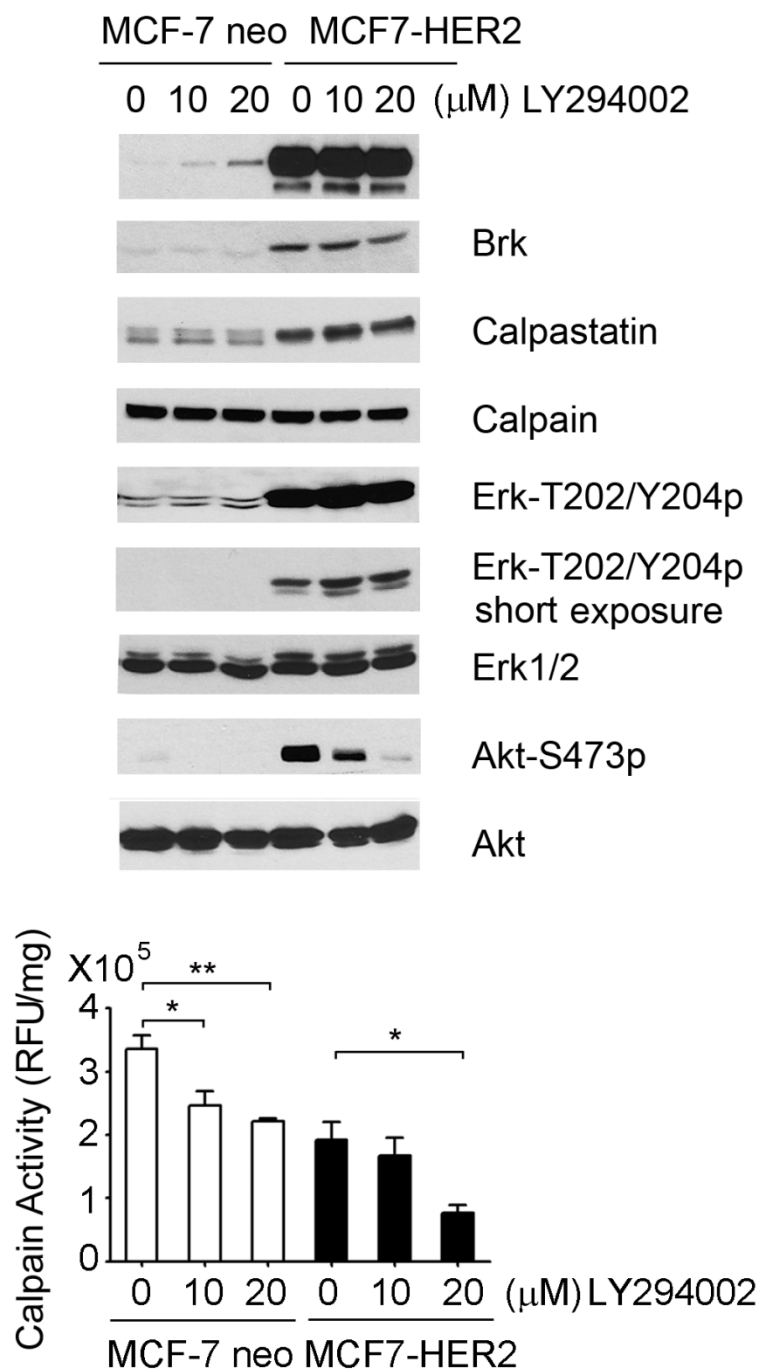
B). HER2 up-regulates calpastatin expression through MAPK pathway. MCF-7 and MCF7-HER2 cells were treated with indicated dose of MAPK inhibitor, PD98059, for 24 hours in 0.5%FBS DMEM. Cell lysates were prepared and analyzed by Western blotting with indicated antibodies. The cells with the same treatment were underwent calpain assay. * $p < 0.05$; ** $p < 0.01$

C). SUM190 cells were treated with indicated dose of MAPK inhibitor, PD98059, for 24 hours in 0.5%FBS DMEM. Cell lysates were prepared and analyzed by Western blotting with indicated antibodies. The cells with the same treatment were underwent calpain assay. ** $p < 0.01$

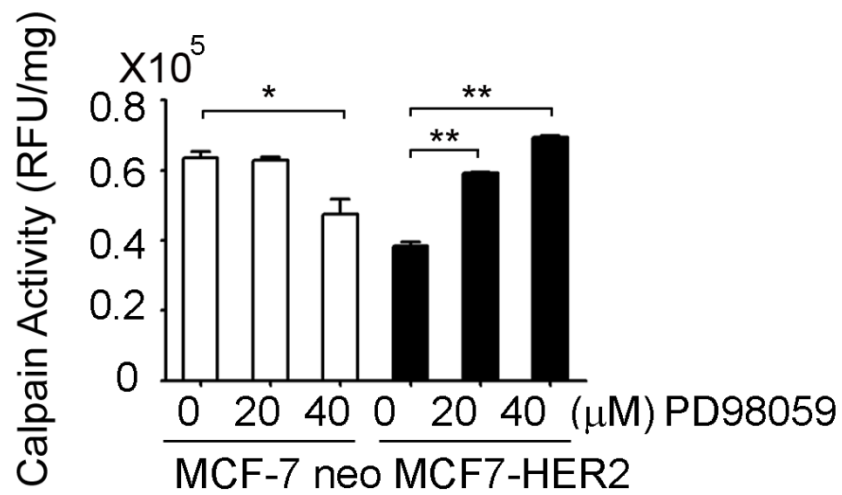
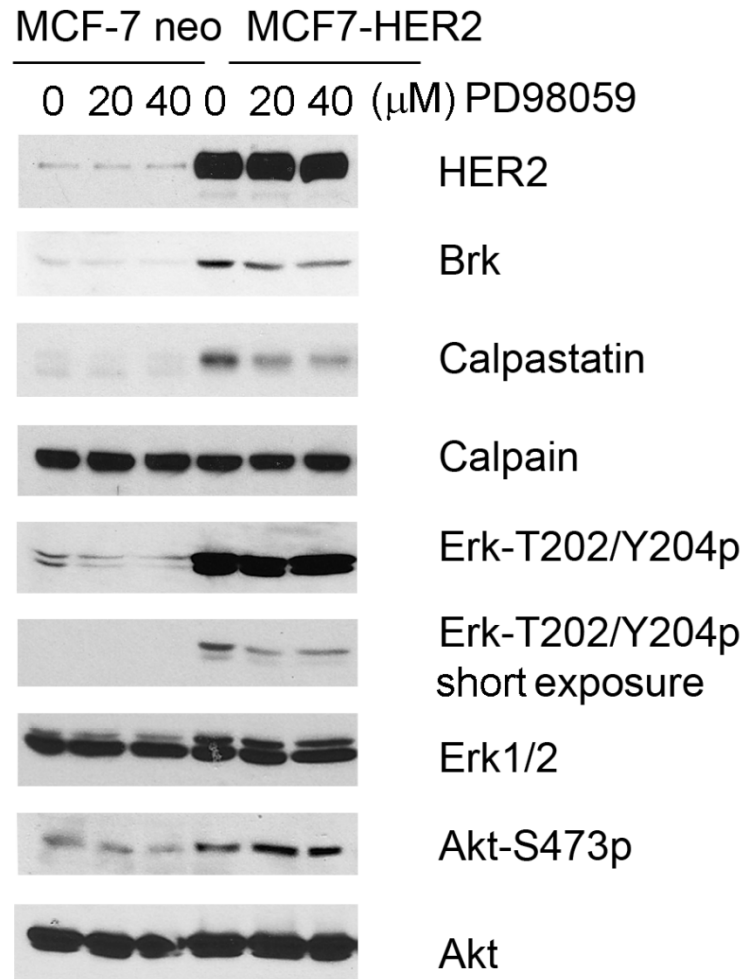
D). SUM 190 were transiently transfected with control siRNA or HER2 siRNA, 24 hours later, the cells with control siRNA and HER2 siRNA were then transfected with control vector and MEK1 vector, in additional 24 hour, cell lysates were prepared and analyzed by Western blotting with indicated antibodies. The cells with the same treatment were underwent calpain assay. ** $p < 0.01$

Figure 8

A



B



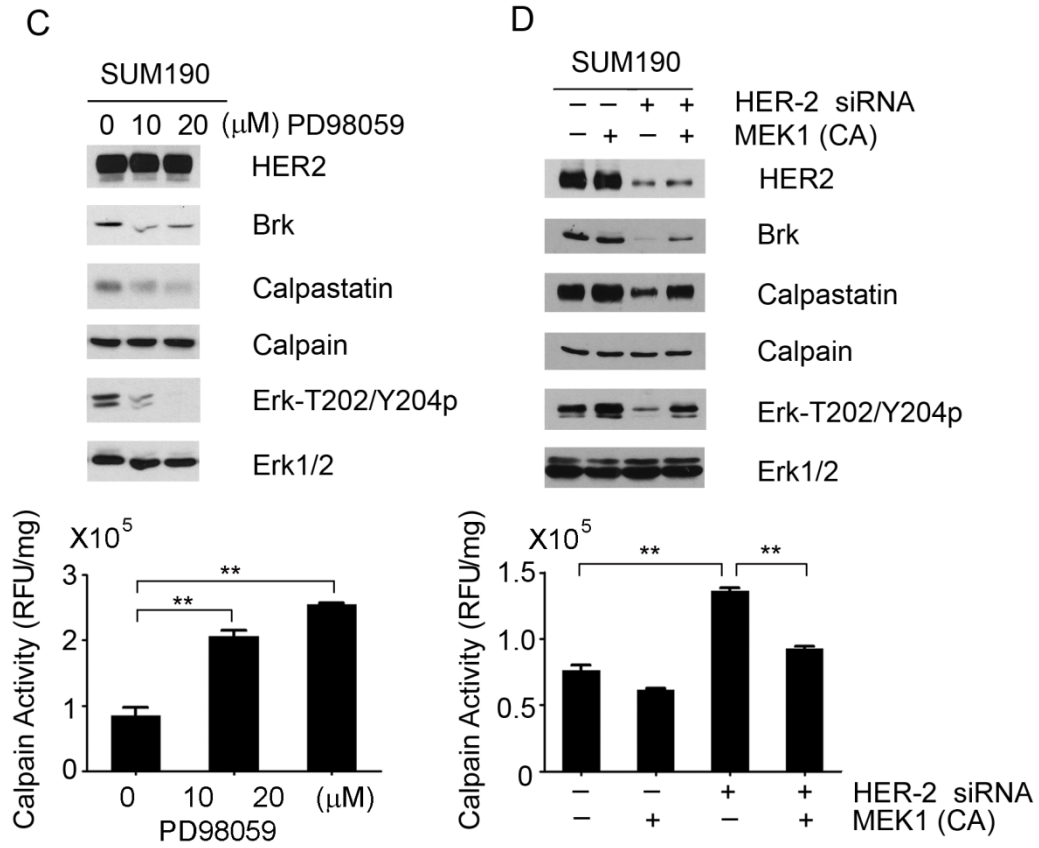
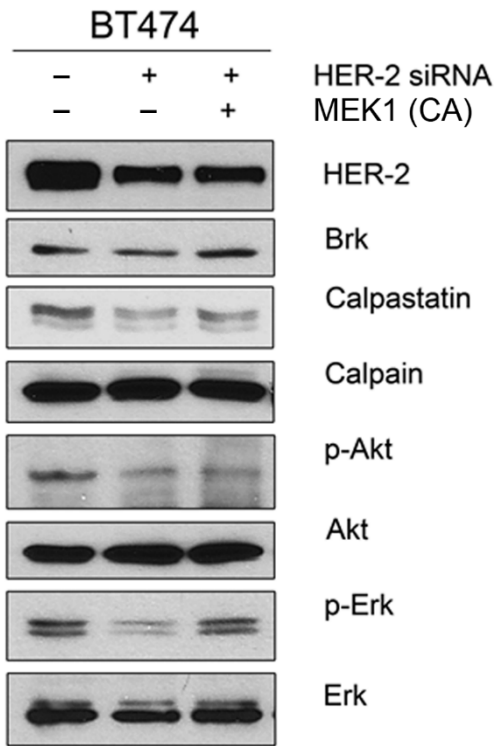
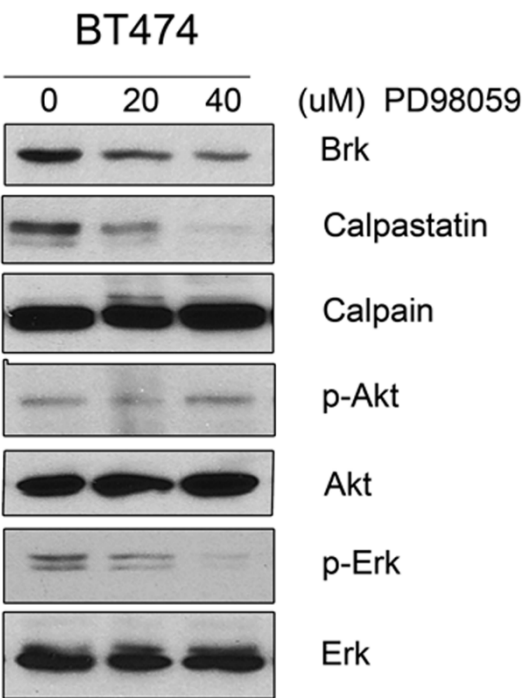


Figure 9. HER2 up-regulates calpastatin expression through MAPK pathway

A). BT474 cells were treated with indicated dose of MAPK inhibitor, PD98059, for 24 hours in 0.5%FBS DMEM. Cell lysates were prepared and analyzed by Western blotting with indicated antibodies.

B). BT474 cells were transiently transfected with control siRNA or HER2 siRNA, 24 hours later, the cells with HER2 siRNA were then transfected with control vector and MEK1 vector, in additional 24 hour, cell lysates were prepared and analyzed by western blotting with indicated antibodies.

Figure 9



Brk is an important mediator of HER2 induced cell proliferation and knockdown of Brk expression sensitizes breast cancer cells to lapatinib treatment

To determine the function of Brk in HER2 mediated biological effects, we knockdown Brk expression through three different lentiviral shRNA infection in SUM190. Abrogation of Brk expression caused 40% reduction in cell proliferation ability as assessed by MTT assay (Fig. 10A). The SUM190 cells harbor an activating mutation (H1047R) in the PIK3CA gene [227], Brk silencing combined with PI3K inhibitor LY294002 induced cell apoptosis in SUM190 as shown by PARP cleavage and apoptosis elisa assay (Fig. 10B). In addition, after silencing of Brk through siRNA the cells demonstrated significant increase of sensitivity to lapatinib treatment (Fig. 10C). As the dose increase of lapatinib, the SUM190 showed decrease of HER2 phosphorylation and knock down of Brk enhanced the decrease of HER2 phosphorylation to lapatinib treatment. Lapatinib treatment did not induced PARP cleavage in SUM190 after Brk silencing but caused cell proliferation inhibition as shown by increase of P27 expression which has been proved involved in cell cycle arrest. Interestingly, lapatinib though did not change Akt phosphorylation, led to remarkable increase of MAPK phosphorylation, Src phosphorylation and Brk protein expression which may be a feedback mechanism of cells in response to lapatinib treatment. The same phenomena was observed in breast cancer cell line HCC1954 too (Figure 11).

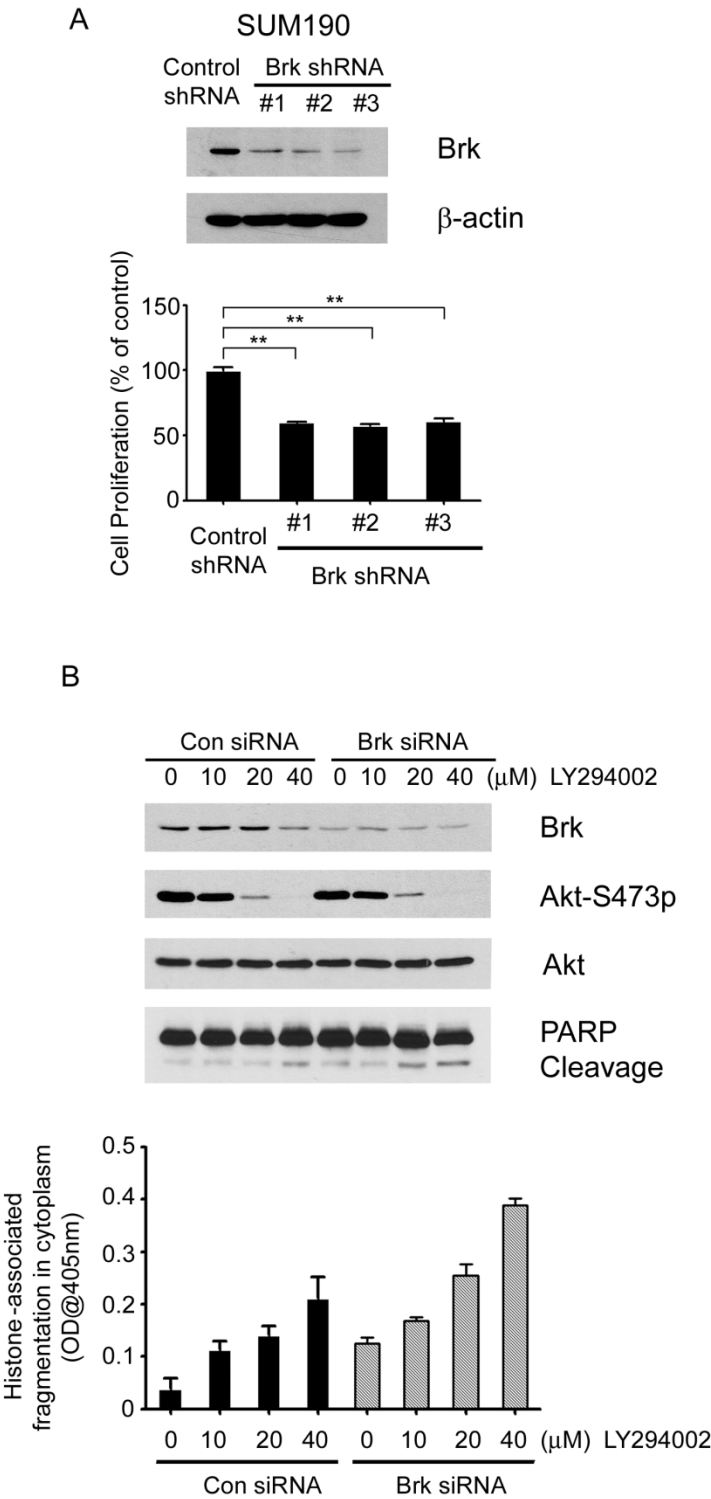
Figure 10. Brk plays an important role in cell proliferation of HER2-overexpressing breast cancer cells and silencing of Brk expression sensitizes the cells to lapatinib treatment

A). Brk plays an important role in cell proliferation of SUM190. SUM190 was infected with three different lentiviral shRNA against Brk. 48 hours later, the stably infected cells were selected with 2 μ g/ml puromycin for a week, Lysates of cells with control shRNA and Brk shRNA were analyzed by Western blotting for indicated antibodies. β -actin served as a loading control. ** $p < 0.01$

B). Knockdown of Brk combined with PIK3 inhibition induced apoptosis in SUM190. SUM 190 were transiently transfected with control siRNA or HER2 siRNA, 24 hour later, the cells were treated with indicated dose of LY294002 in 0.5%FBS DMEM for additional 24 hours for western blot and for an ELISA for quantitative determination of the levels of apoptosis.

C). Knockdown of Brk sensitize SUM190 to lapatinib treatment. SUM 190 were transiently transfected with control siRNA or HER2 siRNA, 24 hours later, the cells were treated with indicated dose of lapatinib in 0.5%FBS DMEM for additional 24 hours for Western blotting assay, and for additional 72 hour for MTT assay.

Figure 10



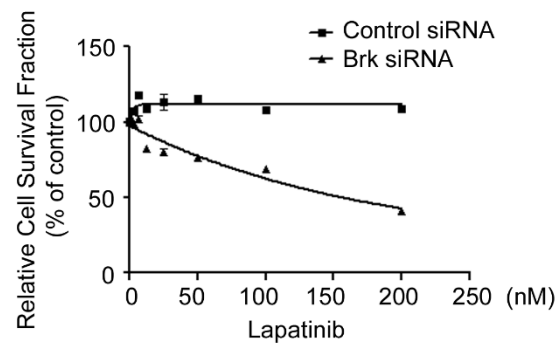
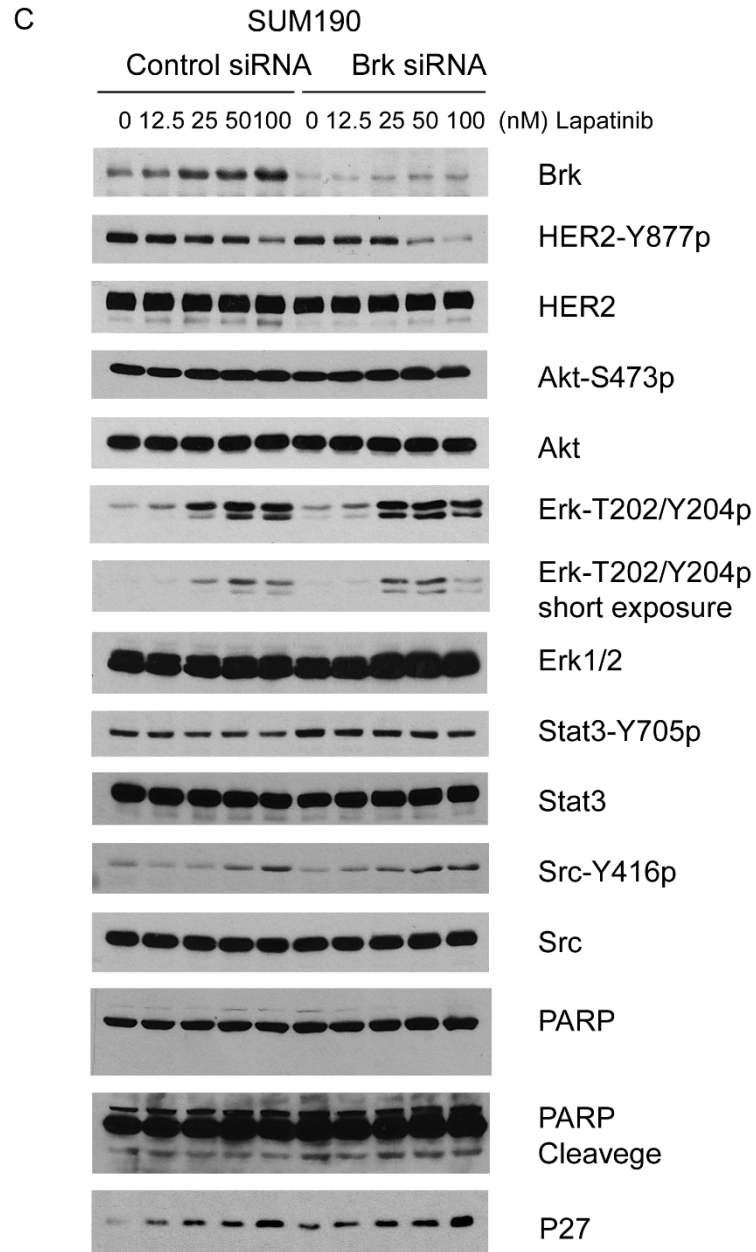
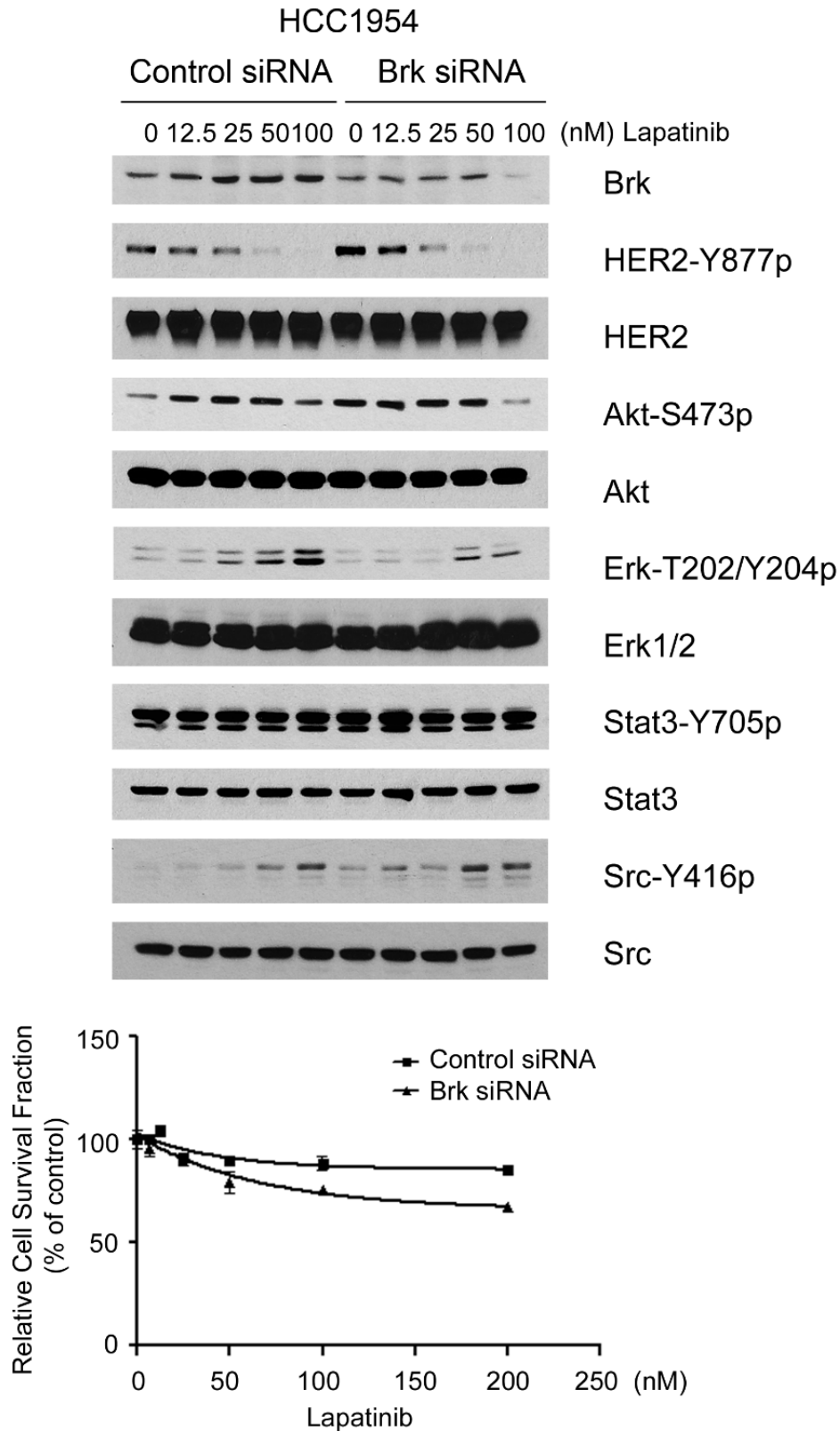


Figure 11. Silencing of Brk expression sensitizes the cell to lapatinib treatment

Knockdown of Brk sensitizes HCC1954 to lapatinib treatment. HCC1954 were transiently transfected with control siRNA or HER2 siRNA, 24 hours later, the cells were treated with indicated dose of lapatinib in 0.5%FBS DMEM for additional 24 hours for western blotting assay, and for additional 72 hour for MTT assay.

Figure 11



Complementary interaction between the expression level of Brk and HER2

To further examine the mutual regulation between Brk and HER2 expression in breast cancer, MCF-7 cell was transfected with incremental dosage of constitutively active (CA) HER2 (V659E) construct. Interestingly, low level of CA-HER2 expression in MCF-7 induced marked increase of Brk expression level, however as the further increase of CA-HER2 expression in the cell, Brk expression showed a trend of reduction (Fig. 12A). In contrast, expression of a constitutively active (CA) Brk (Y447F) in MCF-7 cells also lower the HER2 protein expression (Fig. 12B), and this reduction of HER2 expression demonstrated a CA-Brk dosage dependent manner (Fig. 12C). These results suggest that there is a complementary interaction between the expression of Brk and HER2 although the underlying mechanism is unclear.

Figure 12. Complementary interaction between the expression level of Brk and HER2 in breast cancer cells

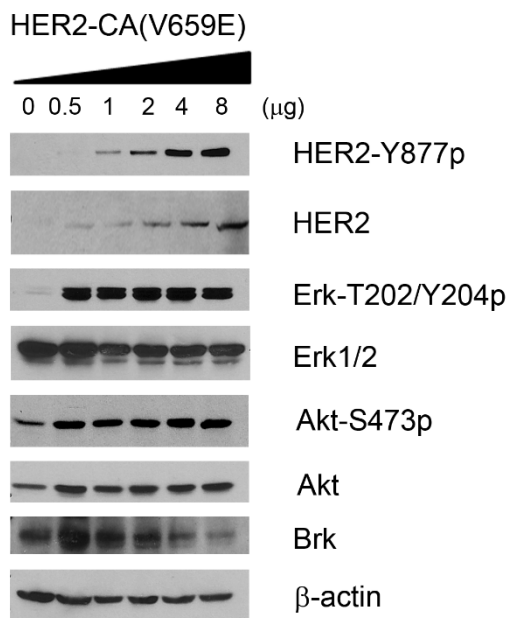
A). MCF-7 cells were transiently transfected with indicated dosage of constitutively active HER2 vectors for 24 hours, cell lysates were prepared and analyzed by western blotting with indicated antibodies.

B). MCF-7 cells were transiently transfected with control vector, wild type Brk, constitutively active Brk (Y447F) and kinase dead Brk (K219M) vectors respectively for 24 hours, cell lysates were prepared and analyzed by western blotting with indicated antibodies.

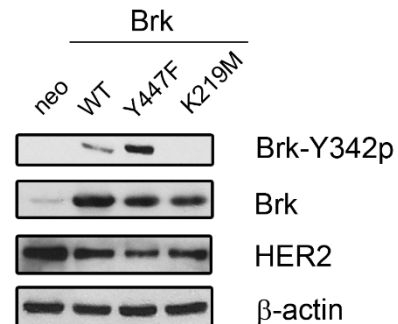
C). MCF-7 cells were transiently transfected with indicated dosage of constitutively active Brk vectors for 24 hours, cell lysates were prepared and analyzed by western blotting with indicated antibodies.

Figure 12

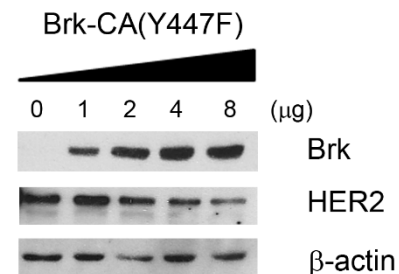
A



B



C



Working model of Brk and HER2 interaction

To sum up, my study validated that HER2 enhances Brk expression via suppressing calpain activation. Overexpression of HER2 activates MAPK pathway resulting in up-regulation of calpastatin expression which then blocks calpain activation. Impairment of calpain activity stabilizes the expression of oncogenic kinases (Brk, Src, Fak and their family members) degraded through calpain pathway. In feedback, over-activation of Brk inhibits HER2 expression, and similarly over-activation of HER2 also inhibits Brk expression (Fig. 13)

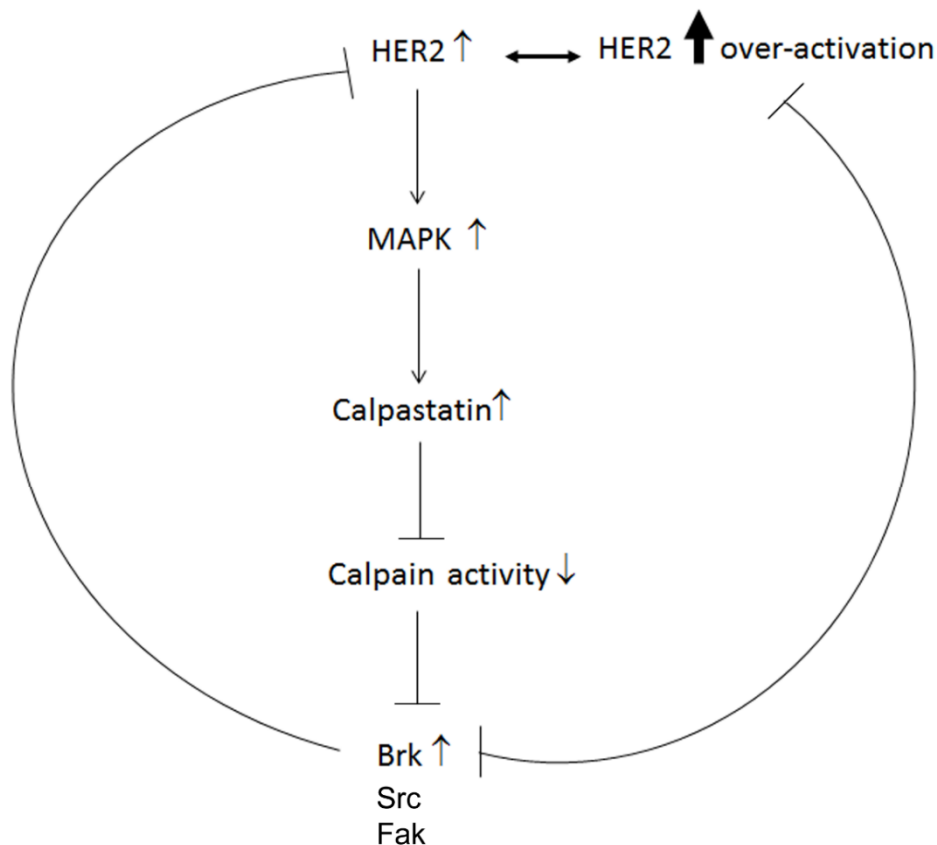


Figure 13. Working model of Brk and HER2 interaction

DISCUSSION

In this paper, we elucidated a novel mechanism by which HER2 and Brk are co-overexpressed in breast cancer and defined the role and biological significance of Brk in HER2-overexpressing breast cancer. We found that there is a significant correlation between HER2 expression and Brk expression in breast cancer cell lines, and overexpression of HER2 increases Brk expression through delaying its protein degradation. Brk can be degraded through a proteolytic pathway involving calpain-I; overexpression of HER2 up-regulates the Brk protein level by inhibiting its calpain-mediated degradation. HER2 inhibits calpain activation via enhancing calpastatin expression which involves the HER2 downstream MAPK signal pathway. We also showed that Brk mediates HER2-induced breast cancer cell proliferation and blocking Brk expression sensitize the cell to lapatinib treatment. Our findings are significant because the data provide a strong mechanistic rationale for developing novel therapies that target Brk to improve the outcomes of anti-HER2 therapy in HER2-overexpressing breast cancer.

Our data showed that overexpression of HER2 in a cell line expressing a low basal level of HER2 enhanced Brk stability, and knockdown of HER2 expression in a HER2-overexpressing breast cancer cell line accelerated Brk degradation. On the basis of these findings, we conclude that HER2 up-regulates the Brk level by inhibiting its degradation. However, the current study does not exclude the possibility that overexpression of HER2 up-regulates Brk through other mechanisms, such as by regulating transcription and/or translation.

Calpain, which includes calpain I and calpain II that differ primarily in their calcium requirements *in vitro* (calpain I requires micromolar Ca^{2+} levels and calpain II requires millimolar levels), belongs to a family of cysteine proteases that have a wide range of cellular functions, including apoptosis, proliferation, and migration [228]. The activity of calpain is strictly regulated by calcium concentrations and interactions with calpastatin (an endogenous calpain inhibitor) [228]. The calpain-calpastatin system, ubiquitously expressed in most tissues of vertebrates and participate in a number of pathological conditions, including hypoxia, ischemia, spinal cord injury, Alzheimer's disease, muscular dystrophy, cataract, and lissencephaly [144-146]. Indeed, there are examples of the activity of calpain being regulated by cell signaling. For instance, c-Myc can stimulate calpain activity by suppressing calpastatin expression [229]. A decade ago, Glanding et al reported that EGF induced activation of calpain in fibroblast that was prevented by inhibition of the Ras-Raf-MEK pathway but not phospholipase C γ signaling pathway [230]. And ERK activation directly phosphorylates and activates m-calpain both *in vitro* and *in vivo* [231]. However, their roles in cancer development have rarely been studied. We showed here for the first time that HER2 over-expression in breast cancer can inhibit calpain enzymatic activity resulting in the up-regulation of oncoprotein like Brk and Src. We demonstrated that the enzymatic activity, but not the expression level, of calpain I is inversely correlated with the HER2 expression level in breast cancer. The mechanism study revealed that HER2 modulated calpain activation through increasing calpastatin expression. To date, how calpastatin expression was regulated remains obscure. Four promoters was found to direct calpastatin expression [232] and analysis of the calpastatin promoter sequence discovered several transcription factor, such as SP1,

AP-1, GATA-1, SRY and NF- κ B binding motifs among the calpastatin promoter region [108]. Our study suggested that MAPK pathway was involved in the modulation of calpastatin by HER2. However, further study is needed to identify which transcription factor directly regulates the expression of calpastatin. Up-regulation of oncoproteins were often observed in HER2 overexpression breast cancer which promoted cancer development cooperating with HER2 and confer resistant to anti-HER2 therapy. Our study shed some light on elucidating the mechanism about co-overexpression of HER2 and many oncogenes in breast cancer. It is reasonable to believe that a new strategy through blocking calpastatin expression to restore calpain activity and accelerate oncoprotein degradation will increase the therapeutic efficiency in HER2 positive breast tumor.

A recent report showed that Brk was degraded through proteasomal pathway in breast cancer and interaction with HSP90 increased Brk protein stability [224]. Consistent with the result, our study confirmed that proteasomal inhibition rescue Brk from CHX induced degradation. Additionally, calpain inhibition also restores Brk expression after protein synthesis inhibition. We further provided direct evidence to prove that Brk is a substrate of calpain I. HER2 increase Brk expression through inhibiting calpain activity suggesting that both ubiquitin-proteasomal pathway and calpain I-mediated proteolytic pathway were responsible for the degradation of Brk.

A recent report showed that experimental overexpression of calpain or activation of endogenous calpain can cleave the cytoplasmic domains of HER2 and contribute to trastuzumab resistance in HER2-positive breast cancer cells [233]. The data from our

study show that overexpression of HER2 can inhibit the activity of calpain. Together, these findings suggest a feedback regulation mechanism inside the cells – HER2 and calpain may have reciprocal roles in regulating each other. While overexpression of HER2 leads to inhibition of calpain activity, when the activity of calpain is suppressed by HER2, calpain may try to overcome the effect of HER2 by cleaving HER2.

In summary, we found that the frequent co-overexpression of Brk and HER2 in breast cancer is regulated by HER2-mediated inhibition of Brk degradation. Brk mediates important oncogenic functions of HER2 in HER2-overexpressing breast cancer cells. Novel approaches to targeting Brk either alone or in combination with anti-HER2 therapy may offer new therapeutic opportunities for improving clinical outcomes of breast cancer treatment.

CHAPTER 3: BRK MEDIATES HER2 INDUCED ONCOGENIC FUNCTIONS

INTRODUCTION

Breast cancer is the most diagnosed form of cancer in women and the second leading cause of cancer deaths in women worldwide. HER2 is gene-amplified and/or overexpressed in 20-30% of breast cancers and has been associated with poor prognosis of breast cancer patients [16, 234, 235]. Previous studies have clearly shown that HER2 plays an important role in breast cancer proliferation, survival, invasion and metastasis [16, 30, 236, 237]. The nonreceptor protein tyrosine kinase Src was shown to cooperate with HER2 to promote the survival and growth of human breast tumor cells and confers resistance to HER2-targeted therapy [238]. It remains interesting, however, to identify additional regulators and mediators downstream of HER2 that are critical for HER2-mediated oncogenic functions of breast cancer [220].

Mammary tumors often display the latent signal transducer and activator of transcription 3 (STAT3) transcription factor constitutively activation, which is required for continuous proliferation and resistance to apoptosis of tumor-derived cells [239-241] and correlates with high expression of the EGFR family members EGFR and/or HER2 [239, 242, 243]. STAT3 can be activated by a wide variety of cytokines and by a number of growth factors and oncogenes, including c-Src, EGFR and HER2 in human cancers [244] STAT3 and STAT5, which plays important roles in cell proliferation and differentiation, are believed to be the substrate of Brk [71, 72]. Brk phosphorylates STAT3 and co-expression of STAT3 and Brk stimulate cellular proliferation [71]. Another Brk substrate, STAP-2(signal transducing adaptor protein-2) was reported

recently to mediate the interaction between Brk and STAT3 [245] and played a crucial role in Brk-mediated STAT3 activation [246].

On the basis of their known individual roles of HER2, Brk and STAT3 in breast cancer invasion and metastasis, in current study, we propose a new paradigm of HER2-Brk-STAT3 triad in regulating breast cancer epithelial mesenchymal transition (EMT) and survival through interaction. Because the majority of studies in literature have used HER2-overexpressing breast cancer cell lines that have been maintained in cell culture for many passages and thus may not faithfully represent the biological role of HER2-Brk-STAT3 triad in the EMT of breast cancer in an effort to identify and determine the molecular players that are critical for HER2-mediated invasion and metastasis in vivo, we designed experiments to recapture HER2-positive breast cancer cells with increased invasive and metastatic potential in nude mice and then compare these cells with parental cells for changes that may account for the enhanced malignant phenotypes. Here, we report our initial results from generation and characterization of such cells and testing our hypothesis on the interaction between HER2, Brk and STAT3 in regulating breast cancer EMT and survival.

MATERIALS AND METHODS [220]

Reagents

Antibodies directed against total Akt, serine 473 (S473)-phosphorylated Akt, threonine 202/tyrosine 204 (T202/Y204)-phosphorylated extracellular signal-regulated kinase (Erk), Y1248-phosphorylated HER2, poly(ADP-ribose) polymerase (PARP), Y705-phosphorylated STAT3 (STAT3-Y705), and total STAT3 were obtained from Cell

Signaling Technology, Inc. Antibodies against total Erk and Brk were purchased from Santa Cruz Biotechnology, Inc. Antibodies against HER2 were purchased from Calbiochem/EMD Chemicals. All other chemicals were purchased from Sigma-Aldrich Corp. The constitutively active STAT3 plasmid was a kind gift from Dr. Suyun Huang's laboratory at The University of Texas MD Anderson Cancer Center.

Breast cancer cell lines and culture

The MCF7, MDA468, BT474 and SKBR3 breast cancer cell lines were originally purchased from American Type Culture Collection. MCF7HER2 cells were created by experimentally elevating HER2, as described previously [221]. All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin and cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Generation of EMT1 subline of MCF7-HER2 cells in vivo

MCF7HER2 cells infected with a recombinant firefly luciferase lentivirus were inoculated into immunocompromised nude mice via tail vein injection (2×10^6 cells in a volume of 0.1 ml serum-free medium). Colonization of these bioluminescent MCF7HER2 cells inside the mice was determined using Xenogen's in vitro imaging system (IVIS). Three of four nude mice inoculated with luciferase-positive MCF7HER2 cells had several IVIS-positive tumors in various parts of their bodies approximately one month after inoculation. These tumors were surgically removed and minced into several small pieces for primary culture in the presence of neomycin (1000 µg/ml), which spares

neomycin-resistance HER2-positive MCF7HER2 cells. The resultant pooled cell sublines were found to be 100% HER2-positive, as measured by fluorescence-activated cell sorting analysis using a HER2-specific antibody. Short tandem repeat profiling by PCR confirmed that the genetic background of the EMT1 subline matched that of MCF7 cells. The animal work was approved by institutional animal care and use committee of M D Anderson Cancer Center.

Western blot analysis

Cells were lysed in a lysis buffer containing 50 mmol/L TRIS-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, 25 µg/mL leupeptin, and 25 µg/mL aprotinin and clarified by centrifugation (14,000 g for 30 min at 4°C). The protein concentration of the cell lysates was determined using the Bradford Coomassie blue method (Pierce Chemical Corp.). Whole-cell lysates were separated by sodium dodecyl sulfate (SDS)-PAGE, transferred onto nitrocellulose by western blotting, and probed with various primary antibodies and horseradish peroxidase–labeled secondary antibodies. The signals were visualized with an enhanced chemiluminescence detection kit (GE Healthcare).

SiRNA/shRNA and transfection

Brk siRNA oligonucleotide duplexes (sense strand: AAGGUGAUUUCUCGAGACAAC dTdT; anti-sense strand: GUUGUCUCGAGAAAUCACCUUdTdT) were purchased from Dharmacon/Thermo Fisher Scientific. Constructs containing STAT3 shRNA (shRNA1 targeting sequence:

GCTGACTACACTGGCAGAGAACTCTTGG; shRNA2 targeting sequence: TGGCTGACTGGAAGAGGCGGCAACAGATT) were purchased from OriGene. Transfection of the siRNA oligonucleotide duplexes and shRNA constructs was performed in a six-well plate (1×10^5 cells/per well) with Lipofectamine 2000 (Invitrogen, Inc.), using the methods recommended by the manufacturer. Using western blotting with specific antibodies, knockdown of Brk with siRNA was examined 48 h after siRNA transfection, and knockdown of STAT3 shRNA constructs was examined 72 h after shRNA transfection.

Immunofluorescent staining of cells

Cells were grown on sterile glass coverslips overnight in a 37°C culture incubator. Prior to immunofluorescent staining, the cells were fixed in pre-chilled -20°C methanol for 5 min and then incubated with 10% normal serum in phosphate-buffered saline (PBS) at 37°C for 30 min to block non-specific binding of IgG. The cells were then incubated with the desired primary antibodies in PBS with 1.5% normal serum at 4°C overnight. After washing the cells twice with PBS, fluorescence-conjugated secondary antibody and 4', 6-diamidino-2-phenylindole (DAPI) were added onto the coverslips, and the cells were incubated in the dark at room temperature for 1.5 h. fluorescently stained cells were examined under a fluorescence microscope.

Transwell chamber assay

Cell migration potential was measured with a Boyden transwell chamber consisting of upper inserts with 8-µm-pore-size filter membranes at the bottom of the

inserts and lower wells in 24-well cell culture plates (Corning Life Sciences). Cells (3.5×10^5 cells in 0.2 mL) suspended in serum-free medium with 0.1% bovine serum albumin were seeded into the inserts of the chambers. The inserts were then placed over the wells filled with 0.5 mL 10% FBS culture medium and incubated in a 37°C incubator for 24 h. Cells that had not penetrated the filter membrane in the inserts were wiped off with cotton swabs, and the cells on the underside of the filter membrane were fixed and stained with the HEMA-3 kit (Fisher Diagnostics). Cells in 10 different microscope fields of each filter were counted. Each treatment group was set in triplicate inserts/wells.

Apoptosis assay

After treatment, the cells were measured for apoptosis using an ELISA kit (Roche Diagnostics Corp.) that quantitatively measures cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) and by western blotting with an antibody that recognizes both un-cleaved and cleaved PARP, as previously described [247] [248].

Cell growth and survival assay

Cells were cultured in 24-well plates with 0.5 ml medium per well at 37°C in a CO₂ incubator. After transient transfection of the cells with Brk siRNA or control siRNA for 48 h, the cells were incubated for an additional 2 h after the addition of 50 µL/well of 10 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were then lysed in a lysis buffer (500 µL/well) containing 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) at 37°C for at least 6 h. The relative number of

surviving cells in each group was determined by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value in each treatment group was then normalized to that of untreated cells as a percentage of the OD value of the control cells and plotted against the treatments.

RESULTS [220]

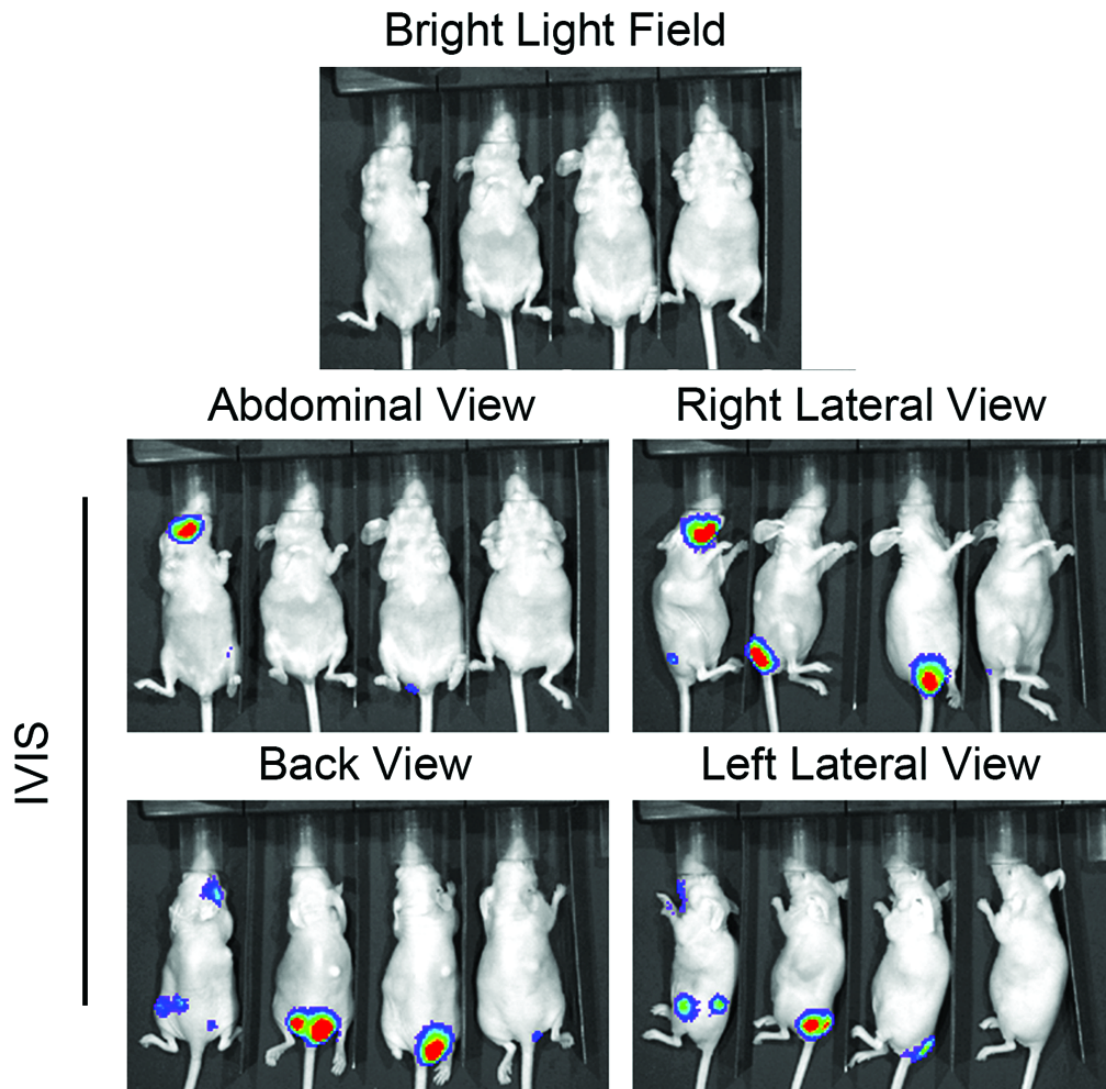
Generation of a metastatic subline of MCF-7 cells overexpressing HER2 (EMT1)

To order to recapture the malignant phenotype of HER2-overexpressing breast cancer cells, we developed several sublines from bioluminescent MCF7-HER2 cell which was infected with a retrovirus containing a recombinant lentiviral construct encoding both green fluorescent protein (GFP) and firefly luciferase (Fluc). The bioluminescent MCF7-HER2 cells were injected into the tail vein of immunocompromised swiss nude mice. We tracked the colonization of the bioluminescent cells inside the body of the mice with the Xenogen's in vitro imaging system (IVIS). Six weeks after injection, several MCF7-HER2 cell colonies were harvested from several remote regions of the mice and expanded after primary culture of tumor tissue obtained at autopsy. Several metastatic sublines of MCF7-HER2 cells were established after several weeks' selection with neomycin (1000 µg/ml) for the presence of neomycin-resistance gene in the HER2 construct. A metastatic subline of MCF7-HER2 cells harvested from the neck region showed the strongest IVIS positivity and underwent fluorescence-activated cell sorting (FACS). The resulting top 5% GFP-positive cell population collected through FACS was expanded by cell culture and termed as EMT1 cells (Fig. 14). FACS analysis confirmed that EMT1 is 100% HER2-positive and short tandem repeat profiling validated that the genetic background of EMT1 subline matches with that of MCF7 cells.

Figure 14. Generation of a metastatic subline of MCF7-HER2 cells (EMT1)

MCF7-HER2 cells cell was infected with a retrovirus containing a recombinant lentiviral construct encoding both Fluc and GFP via the internal ribosome entry site (IRES) mechanism. The bioluminescent MCF7-HER2 cells were injected into the tail vein of nude mice. Six weeks after injection, the tumor was surgically removed and a subline was established by primary culture of the incised tumor tissue. The colonies were selected with G418 (1000 $\mu\text{g/ml}$) for several weeks.

Figure 14



Changes in the phenotype of MCF7-HER2 and EMT1 cells compared with MCF-7 cells

Compared with MCF7neo cells, which show a typical phenotype of epithelial cells that grow tightly connected, the MCF7-HER2 cells, and particularly the EMT1 subline, acquired obvious mesenchymal phenotypes: the cells grew dispersed and were fibroblast-like (Fig. 15A, left columns in the upper and lower panels). Immunofluorescent staining of the MCF7-HER2 and EMT1 cells showed a marked decrease in E-cadherin expression (Fig. 15A., upper panel) and an increase in fibronectin expression (Fig. 15A, lower panel). In addition to the decrease in E-cadherin and increase in fibronectin, Western blotting showed changes in other EMT markers, such as a decrease in β -catenin and increase in N-cadherin (Fig. 15B). Compared with MCF7neo cells, MCF7-HER2 and EMT1 cells also showed increased expression of SNAI 1 but not Twist, two well-known transcription factors that regulate the EMT [249, 250]. Despite the changes in the levels of EMT markers, the EMT1 subline of MCF7-HER2 cells showed substantially increased cell migration potential according to the result of trans-well chamber assay (Fig. 16D). These observations indicate that MCF7-HER2 and EMT1 cells have undergone epithelial-to-mesenchymal transition.

Although HER2 protein level remained similar in EMT1 and MCF7-HER2 cells, the phosphorylation levels of multiple phosphorylation sites of HER2, which are activation-specific, are significantly increased in EMT1 cells (Fig. 15C). These increases were accompanied by remarkable increases in the activation-specific phosphorylation levels of several well-known HER2 downstream substrates, particularly in the levels of phosphorylated STAT3 (STAT3-Y705) and Akt (Akt-S473). Consistent with the finding,

EMT1 cells were more sensitive than MCF7-HER2 cells, whereas MCF7neo cells were insensitive, to treatment with lapatinib, a small molecular HER2/EGFR dual tyrosine kinase inhibitor, as detected by cell growth and survival assays (Fig. 15D, upper panel). The increased sensitivity to lapatinib appears due to the up-regulation in HER2-mediated cell signaling in EMT1 and MCF7-HER2 cells, because they exhibited resistance to doxorubicin, a nonspecific chemotherapeutic agent, , whereas MCF7neo were sensitive to doxorubicin (Fig. 15D, lower panel). These results indicate that the in vivo selection generated a subline of MCF7-HER2 cells with markedly elevated HER2 activity and that the subline cells exhibit a strong mesenchymal phenotype. In addition to the increase in HER2 activity, it was interesting that the levels of total Brk protein and Y342-phosphorylated Brk was significantly increased in MCF7-HER2 cells and moderately increase in EMT1 cells, hinting that there is a reciprocal relationship between HER2 activity and Brk protein level. Meanwhile, the level of activation-specific Y416-phosphorylated Src showed an inverse pattern of change to that of Brk among these three isogenic cell lines, suggesting that a novel role of Brk in substituting Src to cooperate with HER2 for HER2-mediated functions in MCF7-HER2 and EMT1 cells (Fig. 15C).

Figure 15. Phenotype change of HER2-overexpressing breast cancer cells

A). Changes in EMT markers by immunofluorescent staining in MCF7 cells after overexpression of HER2. MCF7neo, MCF7-HER2, and MCF7-HER2/EMT1 cells were immunofluorescently stained with antibodies directed against E-cadherin and fibronectin and counterstained with DAPI. Representative areas visualized under a fluorescent microscope are shown.

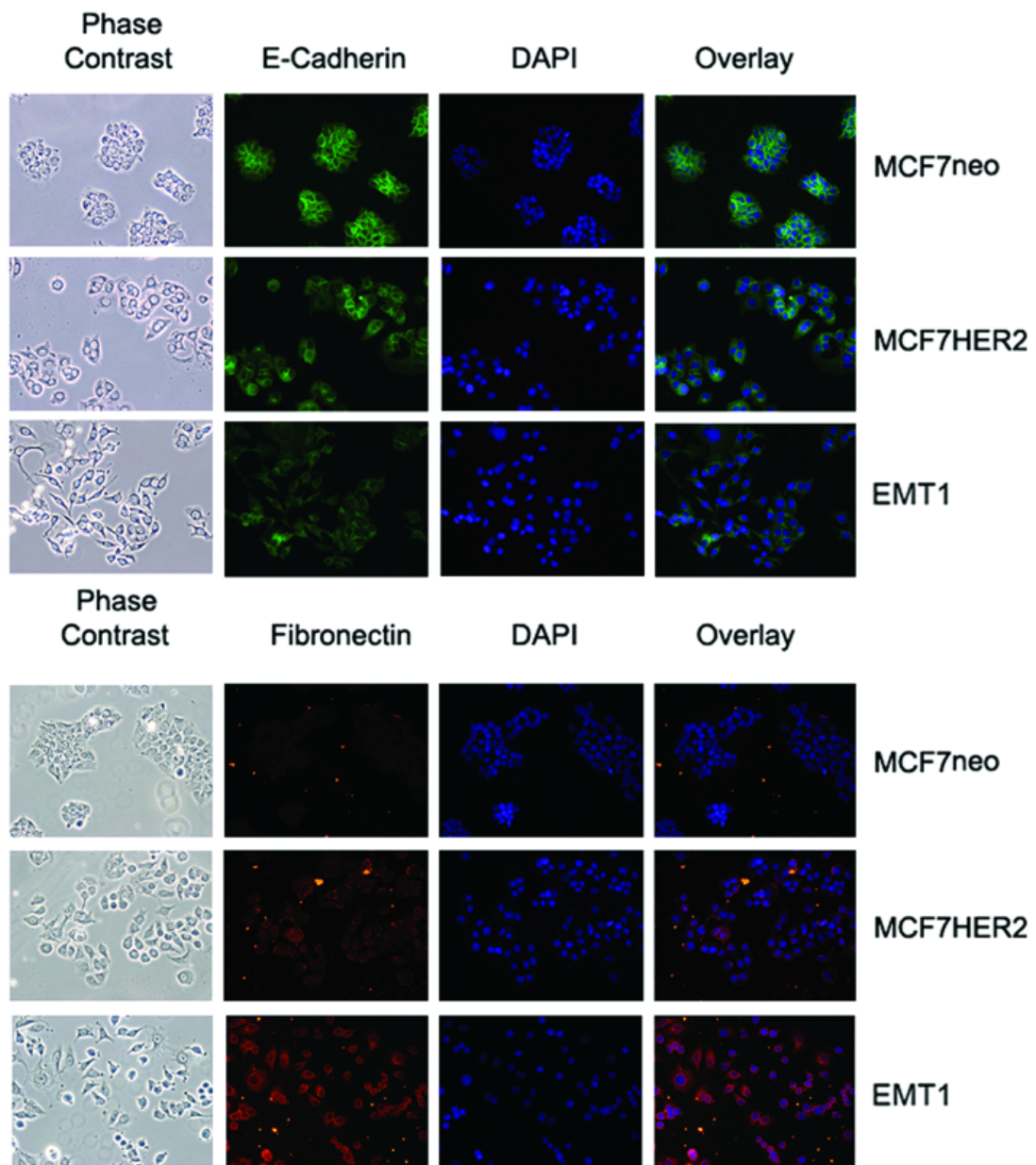
B). Changes in EMT markers by Western blotting in MCF7 cells after overexpression of HER2. Lysates of MCF7neo, MCF7-HER2 and MCF7-HER2/EMT1 cells were analyzed by Western blotting with indicated antibodies.

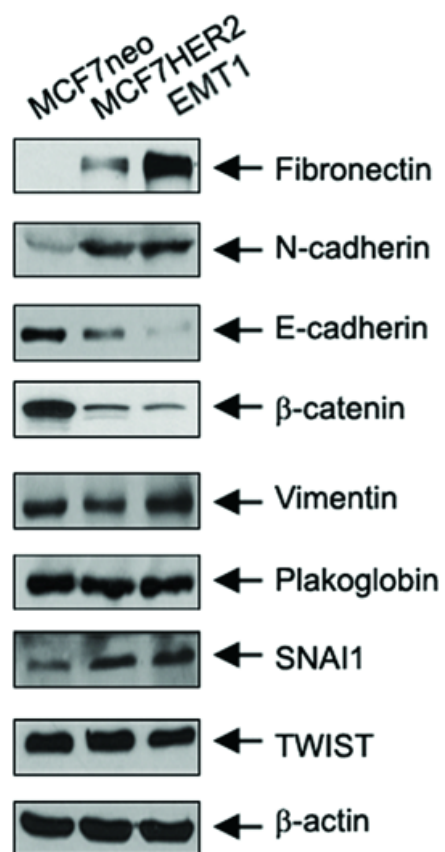
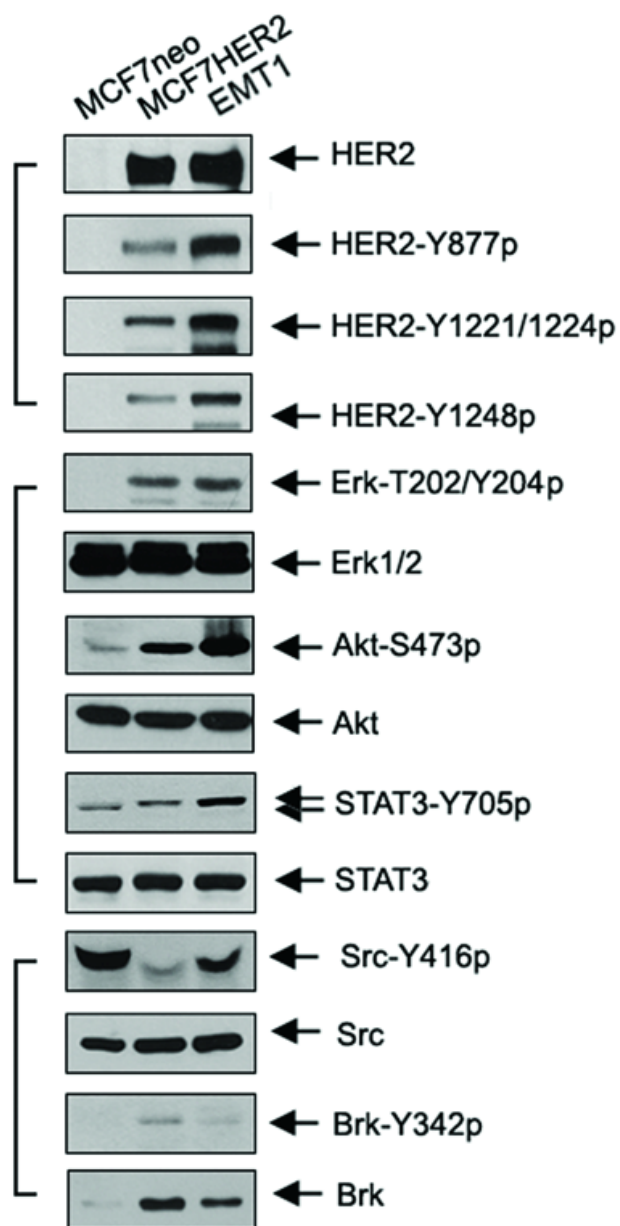
C). Changes in HER2 signaling by Western blotting in MCF7 cells after overexpression of HER2. Lysates of MCF7neo, MCF7-HER2 and MCF7-HER2/EMT1 cells were analyzed by Western blotting with indicated antibodies.

D). Sensitivity of MCF7neo, MCF7heR2 and EMT1 cells to lapatinib and doxorubicin treatment. The cells were treated with indicated concentrations of lapatinib or doxorubicin for 48 h. MTT assays were performed at end of the treatments. The optical density (OD) values of the treated groups were normalized as a percentage of the OD value of untreated or vehicle treated groups of corresponding cell lines. Statistical t-test showed $p < 0.01$ between MCF7neo cells and MCF7heR2 or EMT1 cells treated with lapatinib at all doses; $p < 0.01$ between MCF7neo cells and MCF7HER2 treated with doxorubicin at doses greater than 0.125 nM and between MCF7neo cells and EMT1 cells treated with doxorubicin at doses greater than 1 nM.

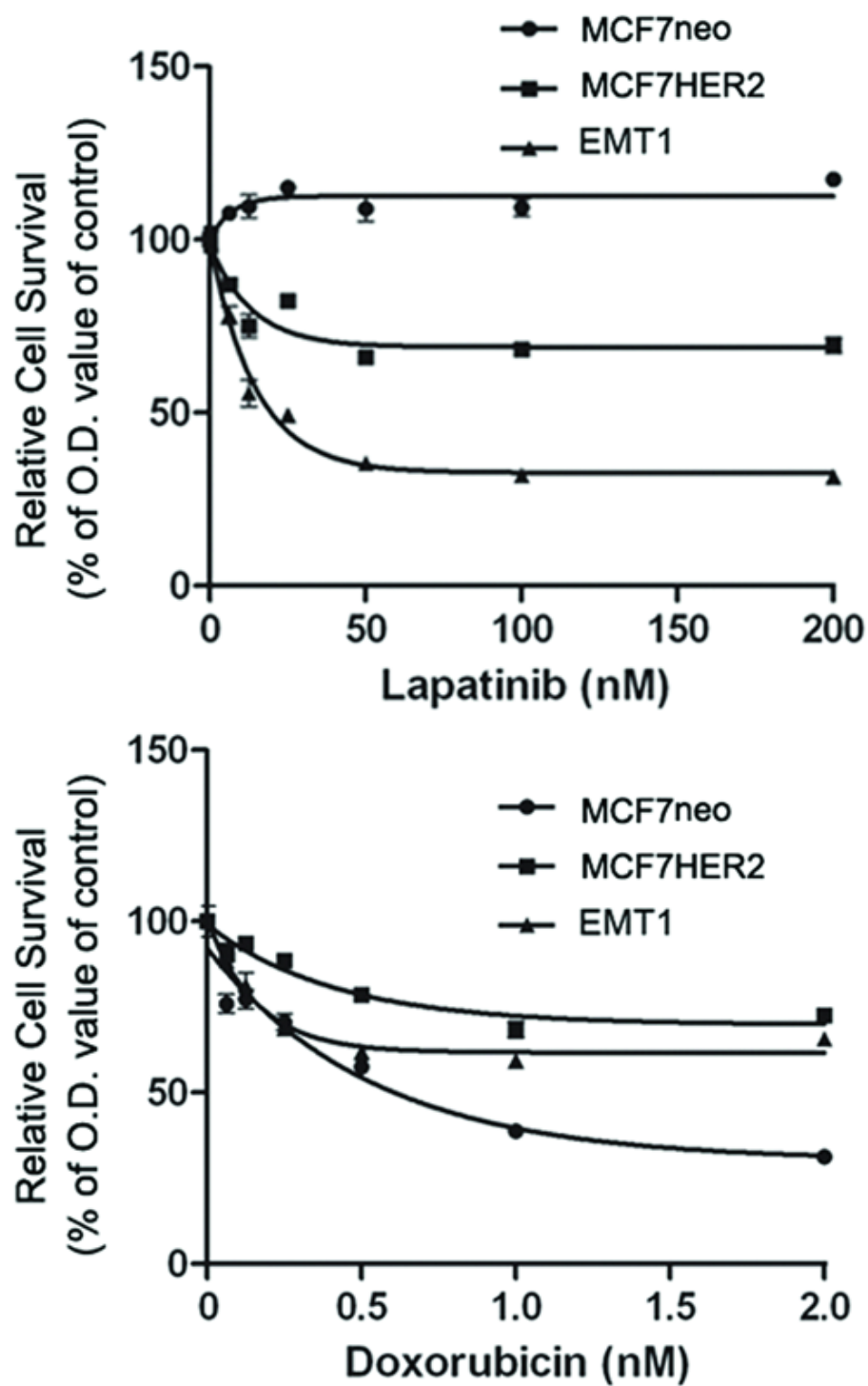
Figure 15

A



B**C**

D



Roles of Brk in HER2-mediated breast cancer cell survival and migration potential

To examine the roles of Brk in HER2-mediated cell signaling and survival and migration potential, we silenced Brk protein expression with small interfering RNA (siRNA) in MCF7neo, MCF7-HER2, and EMT1 cells (Fig. 16A). Firstly, the result of western blot displayed that Brk silencing increased the phosphorylation levels of several HER2 phosphorylation sites (Y877, Y1221/Y1222 and Y1248) in both MCF7-HER2 and EMT1 cells. The basal level of HER2 in MCF7 cells is too low to be detected noticeable changes. Second, knockdown of Brk also led to a slightly compensatory increase in the phosphorylation of Erk (Erk-T202/Y204), but specifically caused marked decline of phosphorylated STAT3 (STAT3-Y705) and Akt (Akt-S473) levels in MCF7-HER2 and EMT1 cells (Fig. 16A). Third, more importantly, knockdown of Brk expression resulted in cleavage of PARP, a marker of apoptosis, in both MCF7-HER2 and EMT1 cells (Fig. 16A). Notably, the induction of PARP cleavage was greater in EMT1 cells than in MCF7-HER2 cells; whereas no obvious PARP cleavage was observed in MCF7neo cells after knockdown of Brk. Further an enzyme-linked immunosorbent assay (ELISA) that quantitatively measures the levels of histone-associated DNA fragmentation in the cytoplasm after induction of apoptosis confirmed that the induction of apoptosis by knockdown of Brk was greater in EMT1 cells than in MCF7-HER2 cells (Fig. 16B). To provide further evidence that knockdown of Brk reduces cell survival, we performed a cell growth and survival assay after transient knockdown of Brk expression in MCF7neo, MCF7-HER2, and EMT1 cells. Transient knockdown of Brk led to a ~15%, ~30%, and ~40% reduction in the numbers of MCF7neo, MCF7-HER2, and EMT1 cells, respectively (Fig. 16C). These findings suggest that EMT1 cell is more dependent on Brk

for survive than MCF7-HER2, though the increase in Brk level was less in EMT1 cells than in MCF7HER2 cells.

A remarkable feature of EMT1 cells is markedly increased migration potential than MCF7neo or MCF7-HER2 cells as shown by Boyden's chamber assay (Fig. 16D). Although MCF7-HER2 cells displayed the changes in EMT markers, its migration potential only slightly increased compared to MCF7neo cells, and parental MCF7neo cells showed minimal level of cell migration potential. The result is consistent with the difference of EMT markers in MCF7neo, MCF7-HER2, and EMT1 cells. However, cell migration potential of EMT1 was significantly decreased after knockdown of Brk expression. Moreover, knockdown of Brk partially reversed changes in EMT markers in EMT1: the epithelial markers (E-cadherin and β -catenin) increased whereas the mesenchymal markers (Fibronectin, N-cadherin) decreased (Fig. 16E), suggesting that Brk mediates cell migration of EMT1 cells. Together, these data indicate that Brk plays important roles in coordinating with HER2 in regulating HER2-positive breast cancer survival and migration potential.

Figure 16. Brk plays important roles in cell migration and survival of HER2-overexpressing breast cancer cells

A). MCF7neo, MCF7-HER2 and EMT1 cells were transiently transfected with Brk siRNA or control siRNA for 48 h. Cell lysates were analyzed by western blotting with indicated antibodies.

B). the same cell lysates in panel A were analyzed by an ELISA for quantitative determination of the levels of apoptosis.

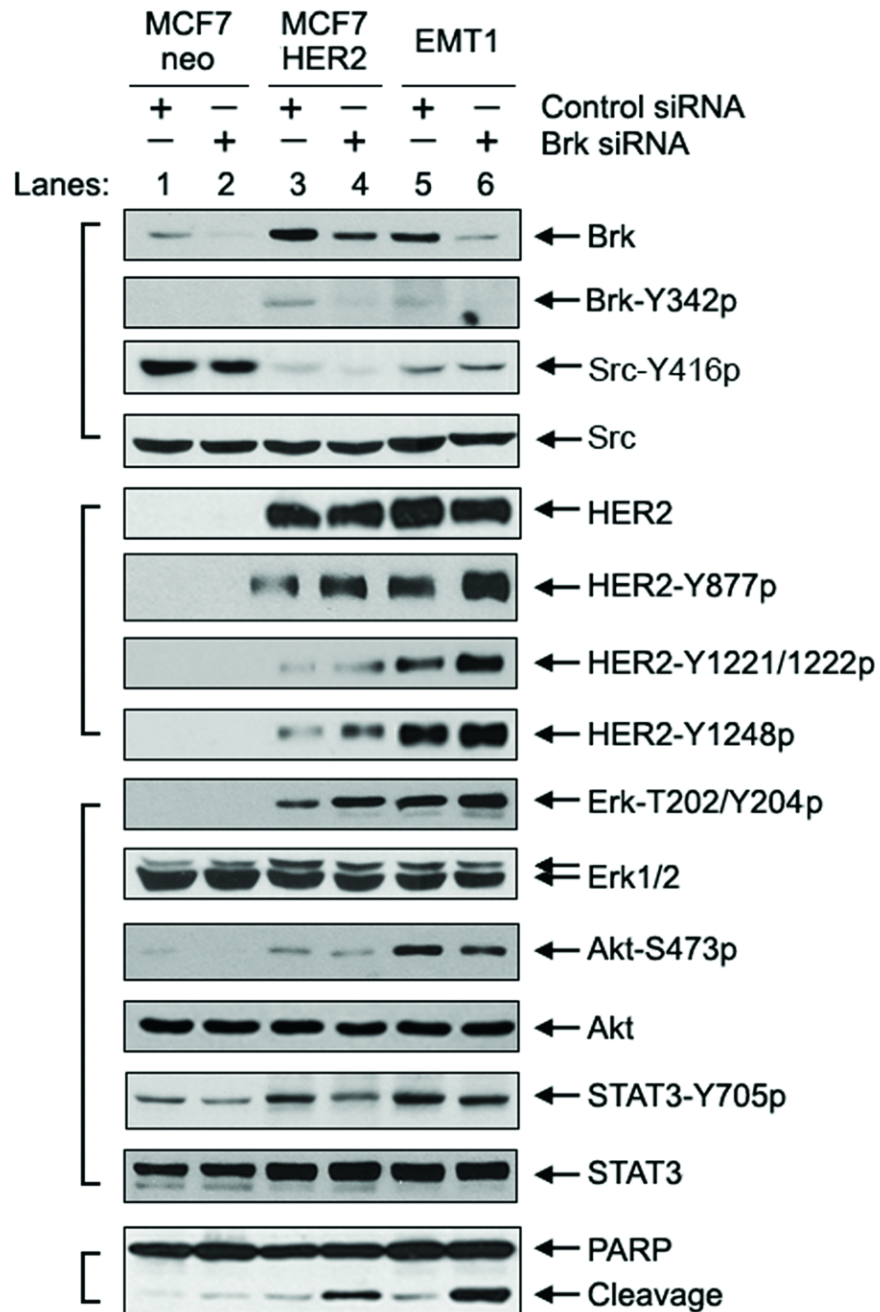
C). MCF7neo, MCF7-HER2, and EMT1 cells were transiently transfected with Brk-specific siRNA or control siRNA as described in panel A. An MTT assay was performed after transfection of the cells with Brk-specific or control siRNA.

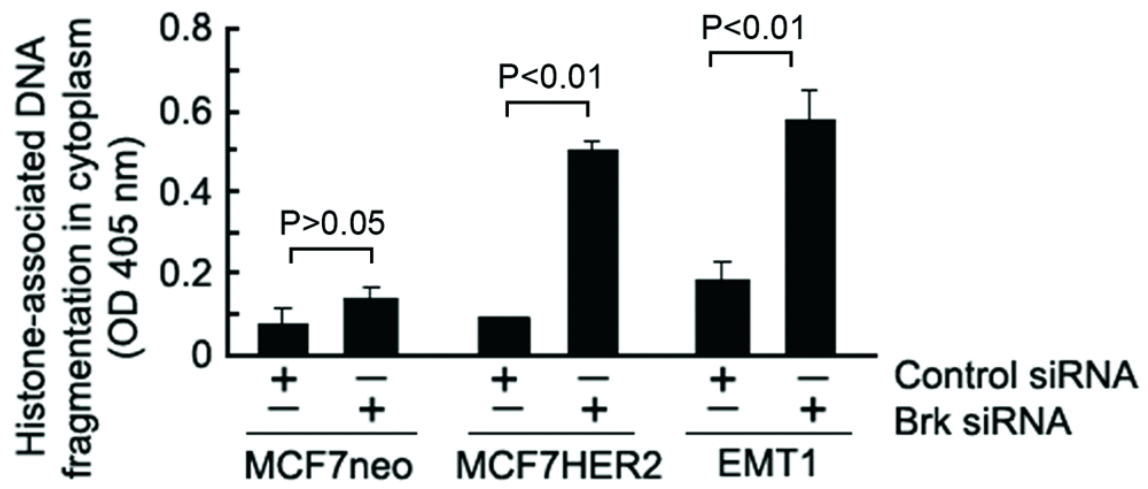
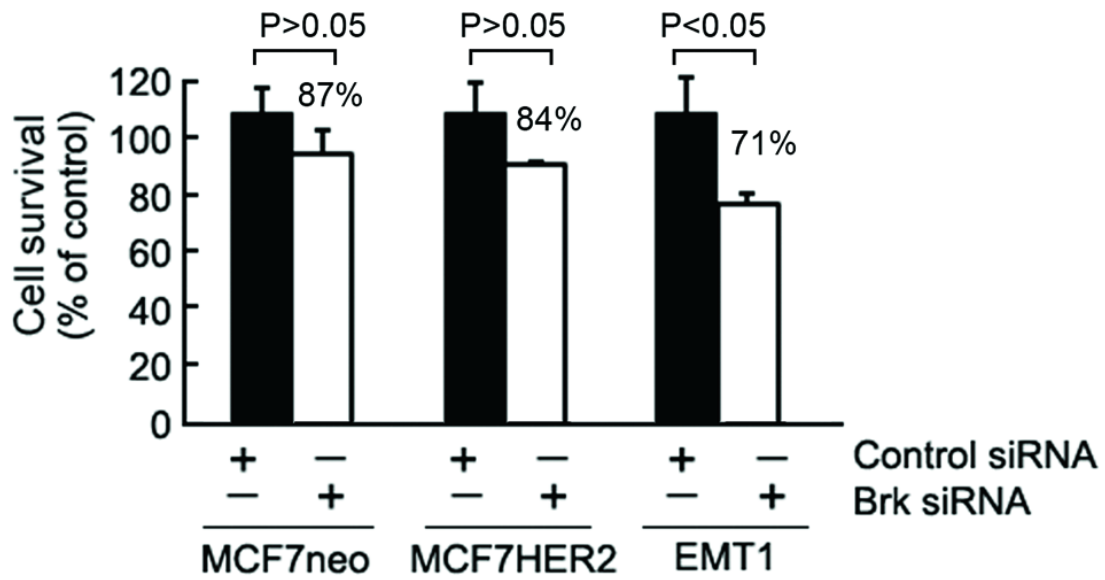
D). MCF7neo, MCF7-HER2, and EMT1 cells were transfected with Brk-specific siRNA or control siRNA for 24 h and then seeded into a Boyden transwell chamber. After incubation overnight, the number of cells that penetrated the transwell membrane per microscopic field was plotted, and representative photomicrographs are shown.

E). EMT1 cells were transiently transfected with Brk siRNA or control siRNA for 48 h. Cell lysates were analyzed by western blotting with indicated antibodies.

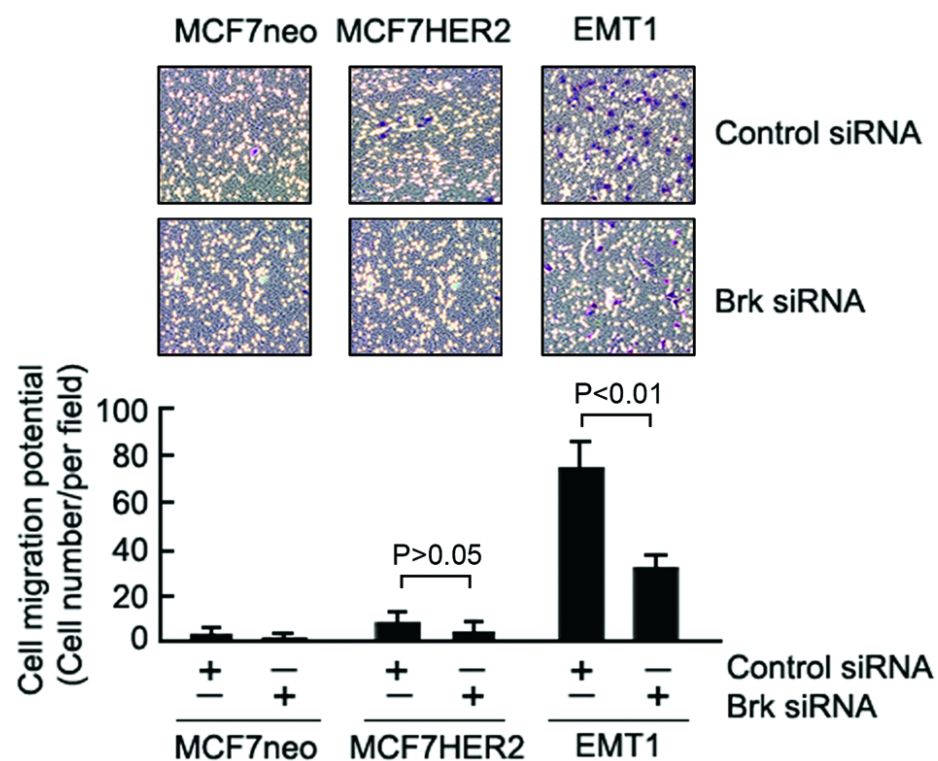
Figure 16

A

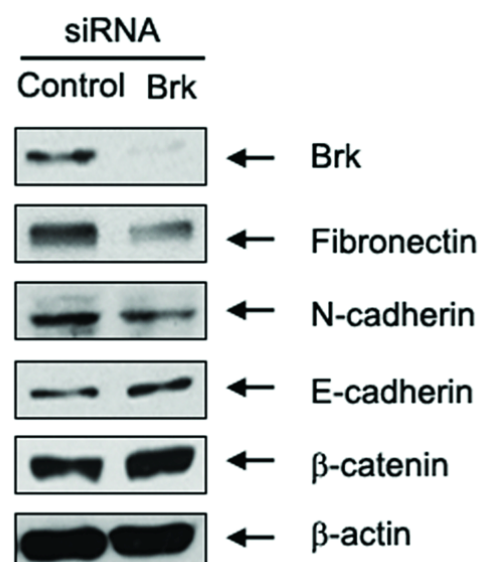


B**C**

D



E



Roles of STAT3 in mediating Brk-regulated HER2 functions

As mentioned, STAT3 is a known substrate of Brk [68]. Many targeted genes regulated by STAT3 are involved in the regulation of cell survival and migration [221, 247-249]. The observations of the marked increase in STAT3 phosphorylation (with no change in STAT3 protein level) in EMT1 cells compared with MCF7HER2 and MCF7 cells, and the abolishment of the increased STAT3 phosphorylation when Brk was knocked down, which was coupled with increased apoptosis and reduced cell migration potential, prompted us to determine a causal role of STAT3 in mediating the role of Brk in EMT1 cells. Figure 17A shows successful knockdown of STAT3 with two distinct small hairpin RNA (shRNA) constructs in EMT1 cells. Knockdown of STAT3 decreased the expression of mesenchymal marker fibronectin protein level and increased the expression of epithelial marker E-cadherin protein level. Silencing of STAT3 expression also induced apoptosis (Figure 17B) and decrease cell migration potential in EMT1 cells (Figure 17C), which is similar to the effect of Brk knockdown (Figure 16B and 16D). Knockdown of STAT3 was also accompanied by changes of EMT markers with a decreased level of fibronectin and an increased level of E-cadherin (Fig. 17A), which suggested that the decrease in the migration potential of EMT1 cells after knockdown of STAT3 is a functional consequence.

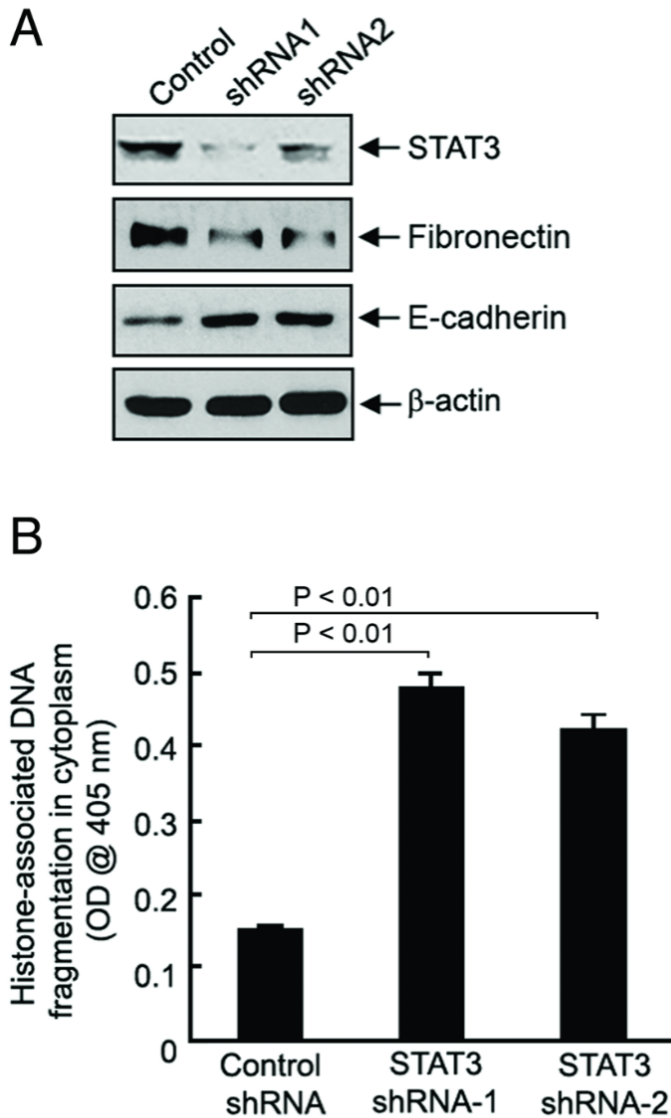
Figure 17. EMT1 depends on STAT3 for migration and survival

A). EMT1 cells were transiently transfected with two STAT3 shRNA or control shRNA for 72 h. Cell lysates were analyzed by western blotting with indicated antibodies.

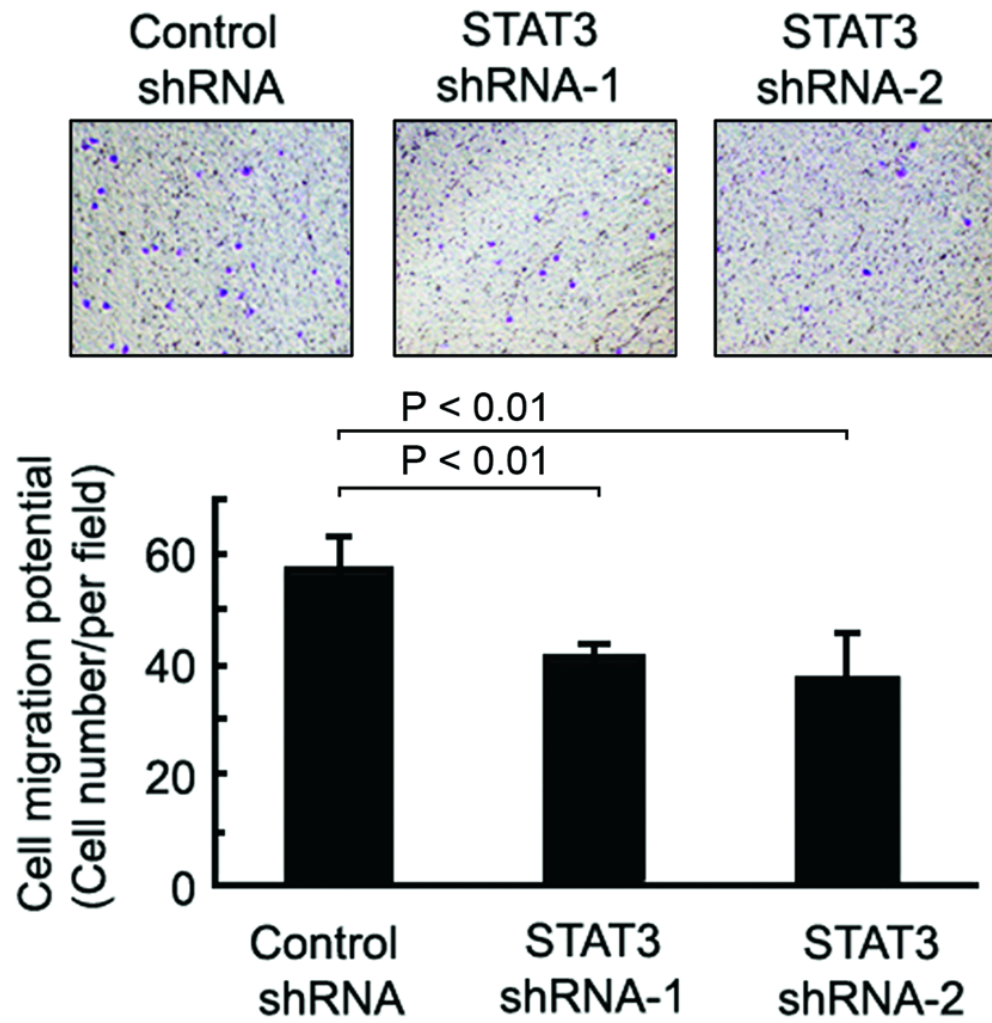
B). the same cell lysates in panel A were analyzed by an ELISA for quantitative determination of the levels of apoptosis.

C). EMT1 cells were transiently transfected with two STAT3 shRNA or control shRNA for 48 h and then seeded into a Boyden transwell chamber. After incubation overnight, the number of cells that penetrated the transwell membrane per microscopic field was plotted, and representative photomicrographs are shown.

Figure 17



C



To confirm STAT3 is an important regulator downstream of Brk signaling to mediate cell survival and migration in EMT1 cells, we transfected a constitutively active STAT3 construct in EMT1 to examine whether it could protect the cells from Brk knockdown-induced apoptosis and inhibition of migration potential. Figure 18A shows that, similar to the findings shown in Figure 16A, knockdown of Brk reduced the level of STAT3 Y705 phosphorylation and induced PARP cleavage in EMT1 cells. The decrease of fibronectin protein level and increase of E-cadherin protein level were also observed. Co-expression of a constitutively active STAT3 along with Brk siRNA led to increase in the level of STAT3 Y705 phosphorylation and reduced apoptosis induced by Brk knockdown as shown by decreased PARP cleavage compared with the effect of Brk-silencing alone (Figure 18A). Independent quantitative apoptosis ELISA testing the level of histone-associated DNA fragmentation in cytoplasm further confirmed the result (Figure 18B). Similar to the effect on protecting cells from Brk knockdown-induced apoptosis, we found that overexpression of the constitutively active STAT3 also restored the migration potential inhibition of EMT1 cells after knockdown of Brk (Figure 18C), as well as prevented Brk knockdown-induced decrease in fibronectin and increase in E-cadherin (Fig.18A).

Figure 18. Restoration of STAT3 activity recovers EMT1 cell migration ability and rescues the cell from apoptosis caused by Brk silencing

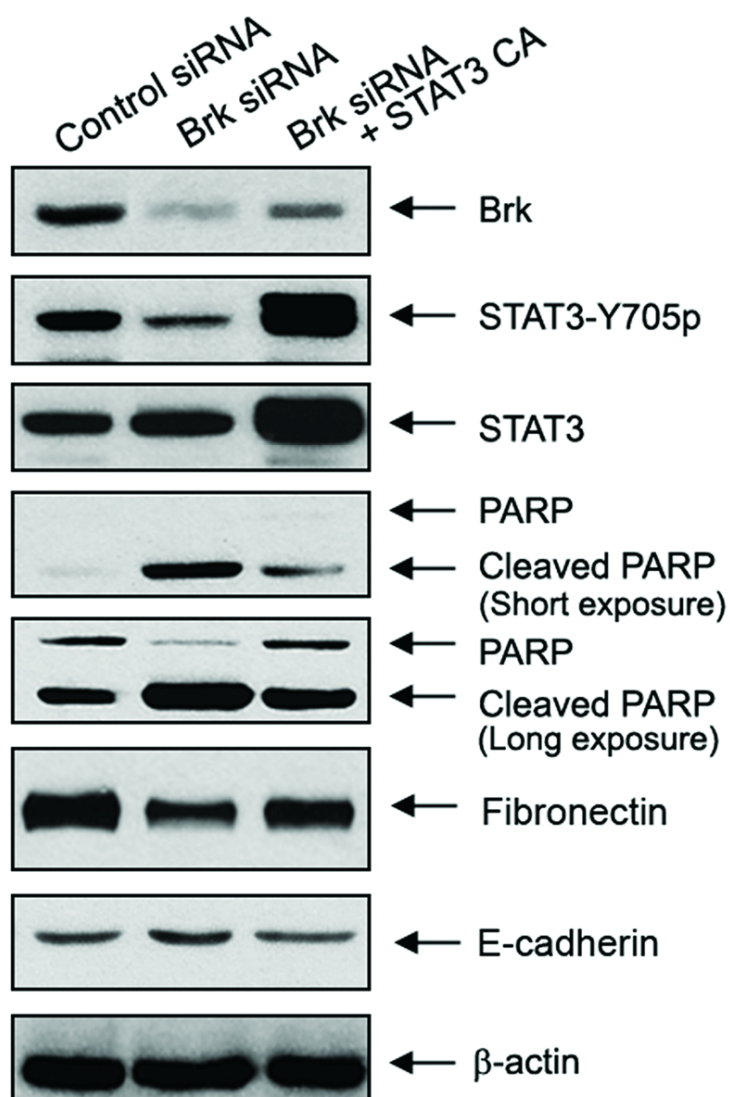
A). EMT1 cells were transiently transfected with control siRNA or Brk-specific siRNA with or without constitutively active STAT3 vector for 48 h, cell lysates were prepared and analyzed by western blotting with indicated antibodies.

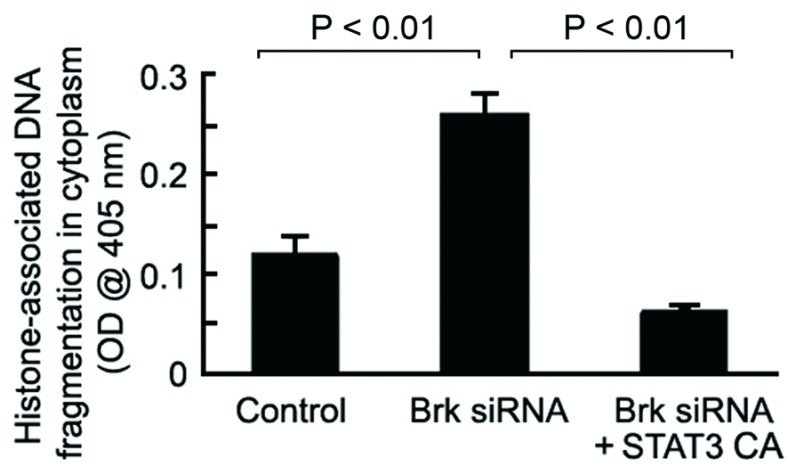
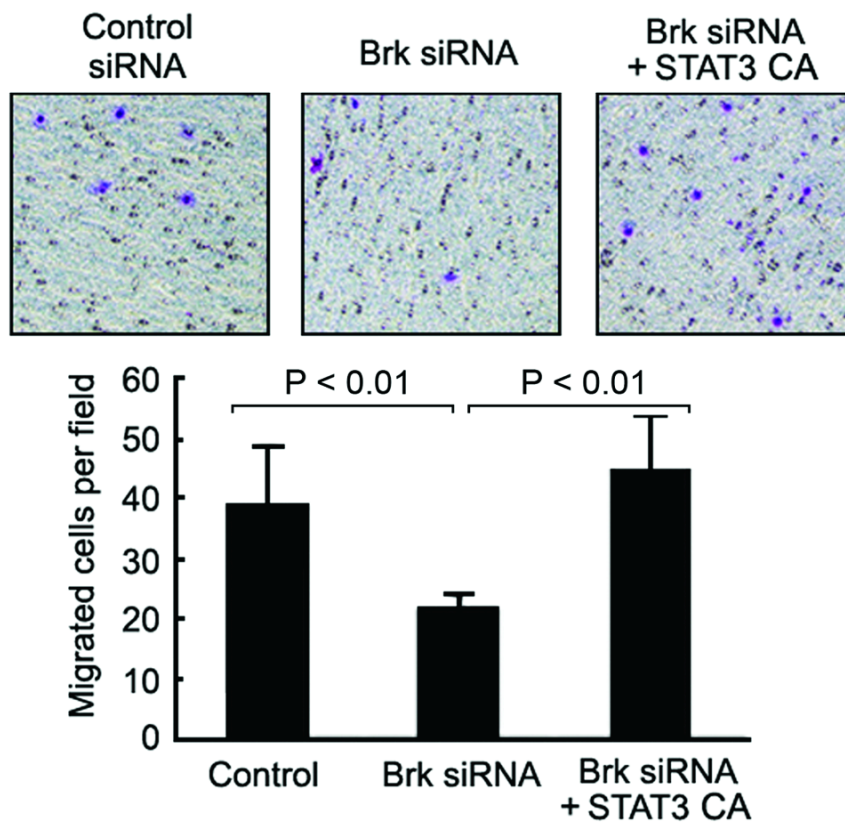
B). the same cell lysates in panel A were analyzed by an ELISA for quantitative determination of the levels of apoptosis

C). EMT1 cells were transiently transfected with control siRNA or Brk-specific siRNA with or without constitutively active STAT3 vector for 24 h and then seeded into a Boyden transwell chamber. After incubation overnight, the number of cells that penetrated the transwell membrane per microscopic field was plotted, and representative photomicrographs are shown.

Figure 18

A



B**C**

Knockdown of Brk induces apoptosis in breast cancer cells with naturally high expression of Brk and HER2

We further examined whether our findings can be observed in other breast cancer cells with naturally occurring high levels of HER2 and Brk. Firstly, we compared HER2 and Brk expression levels in several breast cancer cell lines through western blot and found that BT474 and SKBR3 cells both of which were high HER2-expressing breast cancer cell lines contained higher levels of HER2 and Brk than low HER2-expressing breast cancer cell lines MCF7 and MDA468 cells (Fig. 19A). Knockdown of Brk expression in BT474 and SKBR3 cells induced PARP cleavage and increase in the level of histone-associated DNA fragmentation in cytoplasm (Fig.19B and C). A moderate inhibition of cell migration potential following Brk inhibition was also observed in SKBR3 cells (Fig.19D); however, the difference was less significant compared to the effect found in EMT1 cells. This is probably because, unlike EMT1 cells, which were selected for increased EMT potential, SKBR3 cells have been maintained in cell culture for many years and thus have partially lost migratory potential, like MCF7-HER2 cells (Figure 16D). Likewise, we did not observe significant level of cell migration of BT474 cells due to the low migration potential of the cells when measured by the Boyden's chamber assay.

Figure 19. Brk silencing induces cell apoptosis and migration potential decrease in naturally overexpressed Brk and HER2 breast cancer cell lines

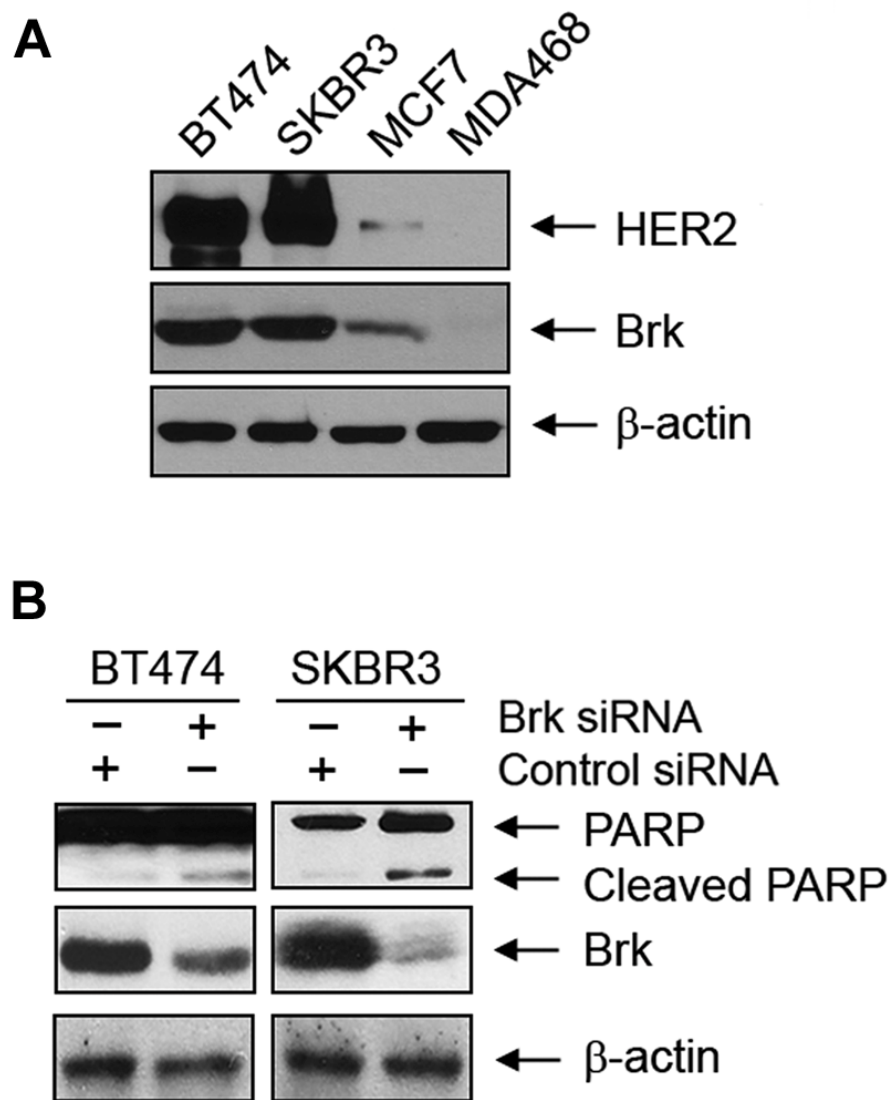
A). Cell lysates of BT474, SKBR3, MCF-7 and MDA 468 were analyzed by Western blotting with indicated antibodies.

B). BT474 and SKBR3 cells were transiently transfected with Brk siRNA or control siRNA for 48 h. Cell lysates were analyzed by western blotting with indicated antibodies.

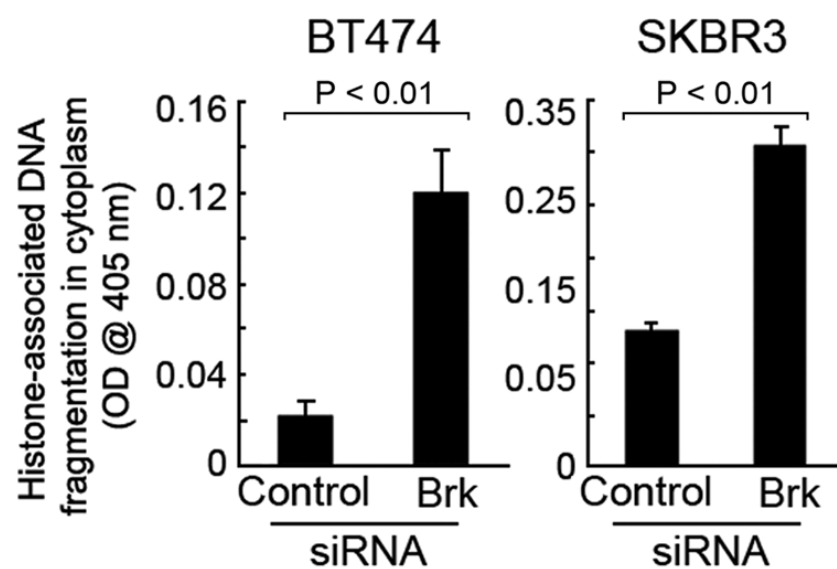
C). The same cell lysates in panel B were analyzed by an ELISA for quantitative determination of the levels of apoptosis.

D). SKBR3 cells were transiently transfected with Brk siRNA or control siRNA for 24 h, and then seeded into a Boyden transwell chamber. After incubation overnight, the number of cells that penetrated the transwell membrane per microscopic field was plotted, and representative photomicrographs are shown.

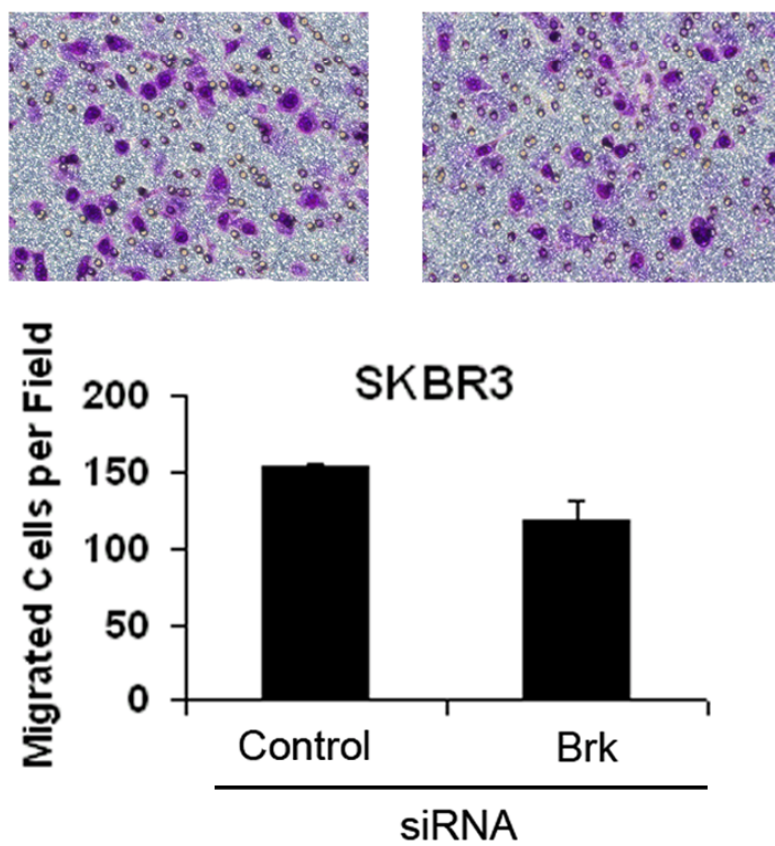
Figure 19



C



D



In summary, our data showed that Brk mediates cell survival and migration potential in HER2-overexpressing breast cancer cells through STAT3 pathway.

DISCUSSION

In current study, we derived a useful subline (EMT1) with increased EMT phenotype from MCF7 breast cancer cells transfected with high level of HER2. Compared with MCF7-HER2, EMT1 cells maintain a similar level of HER2, but exhibit markedly higher basal levels of activation-specific phosphorylation of HER2, Erk, STAT3 and Akt. We found that the nonreceptor tyrosine kinase Brk was up-regulated following HER2 overexpression and plays an indispensable role in conferring the EMT phenotypes and maintaining cell survival in this cell model. We further demonstrated that STAT3, a known substrate of Brk, plays a critical role in mediating the functions of Brk. We confirm the role of this HER2-Brk-STAT3 triad in regulating the survival of breast cancer cells with naturally occurring high level of HER2.

Our previous study indicated that HER2 up-regulates Brk through inhibiting its calpain mediated protein degradation. However, over-activation of HER2 results in the decrease of Brk protein expression. We consistently found in our repeated experiments that the basal levels of phosphorylated HER2 on several sites were higher in EMT1 cells than in MCF7HER2 cells, but the level of increase in Brk was moderately less in EMT1 cells than in MCF7-HER2 cells, strongly suggesting that the two tyrosine kinases are somewhat complementary in functions. Furthermore, while the level of Brk was up-regulated by HER2, knockdown of Brk increased HER2 phosphorylation on multiple sites. This phenomenon was seen in both MCF7-HER2 and EMT1 cells. Because

knockdown of Brk led to cell death via apoptosis, and also reduced cell migration potential in the case of EMT1 cells, these findings suggest a model wherein the Brk is upregulated and required by HER2 for mediating the functions of HER2; when this link was disrupted, cells underwent apoptosis; as an attempt to rescue the cells from undergoing apoptosis, HER2 exhibits an increased activity. However, increased phosphorylation of HER2 appeared futile because the cells underwent apoptosis and the levels of phosphorylated STAT3 and Akt were markedly inhibited as results of knockdown of Brk. It is unclear from our current study how the levels of HER2 phosphorylation were increased when Brk was knocked down. Because the increase in HER2 phosphorylation occurs at the multiple sites, involvement of a third tyrosine kinase or kinases that can phosphorylate these sites on HER2 is less likely. Thus, it may be related to the mechanisms underlying HER2 homo or heterodimerization with other HER family proteins, which are known mechanisms leading to HER2 phosphorylation.

Currently, whether overexpression of HER2 alone can drive EMT in breast cancer is still controversial. In our study, we found that overexpression of HER2 in MCF7 cell induced cell morphology changes from epithelial phenotype to mesenchymal phenotype accompanying EMT marker change; however, the cell migration capability was not significantly increased in these cells. We found a cycle of cell selection *in vivo* rendered the cells to be highly migratory measured by the Boyden's chamber assay, suggesting that increases and/or involvement of additional molecular players are needed for the EMT phenotype. A notable difference between MCF7HER2 and EMT1 cells is an increased dependence of the cells on Brk, which can be relieved in large by expression of constitutively active STAT3. Both Brk and STAT3 play important roles not only in cell

survival but also in cell motility [77, 78, 251]. Thus, STAT3 may be an important molecule in mediating the functions of HER2 and Brk, forming a triad.

STAT3 is a known substrate of Brk and overexpression of constitutively active STAT3 protects EMT1 cells from Brk knockdown-induced cell apoptosis and decrease of cell migration indicating that Brk mediates the interaction between HER2 and STAT3. A previous study exploring the mechanisms underlying activation of STAT3 in breast cancer found that level of activated STAT3 was not directly positively linked to the level of HER2 in breast cancer specimens [252]. The results of our current study suggest that HER2 may regulate the activation of STAT3 via Brk and the increases in HER2 phosphorylation on multiple sites after knockdown of Brk were not accompanied by an increase in the level of phosphorylated STAT3 and Akt but it did elevate that of Erk. This may explain the reported lack of significant correlation between activated STAT3 and HER2 in the specimens from breast cancer patients [252].

In addition, in our cell model we found that overexpression of HER2 in MCF7 cells up-regulated the levels of Brk total protein and activation-specific Brk Y342-phosphorylation whereas reduced the level of activation-specific Src Y416-phosphorylation. Although the underlying mechanisms were not explored in our study, the finding suggests that Src and Brk have complementary functions too. Src has been shown to be an important co-target for HER2-targeted therapy for breast cancer [238]; our result suggests that Brk might be another ideal co-target for HER2-targeted therapy for breast cancer. Because EMT1 cells were more sensitive than MCF7HER2 cells to Brk knockdown-induced apoptosis and inhibition of cell migration, these results indicate that Brk plays an essential role in mediating the observed phenotypes acquired by EMT1 cells.

In summary, our current study depicted a new paradigm supporting a role of HER2, Brk and STAT3 triad in regulating breast cancer EMT and survival via complicated signaling network. Brk may be a molecular target suitable for developing novel therapeutic approaches for breast cancer.

CHAPTER 4: INDUCIBLE BRK TRANSGENIC MICE

INTRODUCTION

Brk (breast tumor kinase) also called protein tyrosine kinase 6 (PTK6) is a ~ 56-60 kDa nonreceptor protein kinase cloned in 1994 in a screen for tyrosine kinases expressed in a metastatic breast tumor [56]. Sik (Src-like intestinal kinase) is the murine Brk-ortholog which shares 80% identity with Brk and was cloned from the small intestine and skin [253]. Overexpression of Brk is observed in up to 86% of invasive ductal breast carcinomas [57, 86]. Brk overexpression is associated with the carcinoma content of breast tumors [86], breast tumor grade [254] and invasiveness of breast cancer cell lines [78]. Brk is also expressed in some normal tissues including the intestinal epithelium, melanocytes, keratinocytes [55, 253], prostate luminal epithelium [255], and lymphocytes [60], but not in normal mammary tissue [59]. The universal high expression of Brk in breast cancer suggests that Brk plays important roles in breast tumor development. An analysis of purified Brk protein showed that Brk is regulated by autophosphorylation and autoinhibition mechanisms [63]. Brk activity is increased by autophosphorylation at tyrosine 342 in the activation loop of its kinase domain [63]. Mutation of tyrosine 447 (Y447F), which is analogous to tyrosine 527 of Src, leads to constitutive activation of Brk owing to the accessibility of the SH2 domain [63]. In contrast, mutation of lysine 219 (K219M) completely abolishes the kinase activity of Brk [66].

Mouse models have been extensively used for studying the genetic basis of breast cancer [256]. Transgenic mouse model carrying unactivated or activated HER2/neu under the transcriptional control of the mouse mammary tumor virus promoter/enhancer

(MMTV) have been used for two decades [183, 193]. Whereas rapid tumor progression is observed in transgenic mice carrying the activated HER2/neu transgene, development of focal mammary tumors has a long latency in mice with unactivated HER2/neu in the mammary epithelium; however, many of the tumor-bearing transgenic mice with unactivated HER2/neu developed secondary metastatic tumors in the lungs [193]. Transgenic mice expressing transforming growth factor alpha (TGF- α), an important ligand of EGFR, in the mammary epithelium also develop spontaneous focal mammary tumors after a long latency [257]. Bitransgenic mice that co-express TGF- α and HER2/neu in the mammary epithelium developed multifocal mammary tumors that arose after a significantly shorter latency period than was observed in either parental strain (mice with expression of TGF- α alone or HER2/neu alone) [258]. Inhibition of EGFR with a small- molecular tyrosine kinase inhibitor (AG-1478) markedly delays breast tumor formation in MMTV-HER2/neu+ MMTV-TGF- α bitransgenic mice [259]. These results indicate that EGFR-mediated cell signalling is important in HER2/neu-mediated tumorigenesis.

Several inducible transgenic mouse models have been developed for studying mammary-specific transgene expression in mice [260-264]. MTB transgenic mice [262] express the reverse tetracycline-dependent transactivator (rtTA) in the mammary epithelium(MMTV-rtTA) and are particularly useful for doxycycline-induced transgene expression [198, 262]. Responder constructs are generated using the TMILA plasmid, which contains seven tandem Tet operator sequences upstream of a multiple cloning site, followed by an internal ribosome entry site (IRES) and the firefly luciferase coding region [265]. The inclusion of luciferase is an innovative feature that allows for easy

detection of transgene expression by using an in vivo imaging system (IVIS) to monitor the development of target gene expressing tumors in live animals.

In this study, I generated the first mammary gland specific Brk Tet-on inducible mice by crossing transgenic mice carrying a single copy of a Brk transgene with MMTV promoter-driven reverse tetracycline-controlled transactivator mice (MTB). I use this bitransgenic mouse (referred to as MMTV-rtTA/Tet-O-Brk) model to observe whether Brk induction in the mammary gland will induce breast tumorigenesis. I also crossed the bitransgenic mice with MMTV-neu transgenic mice [193] to generate the tripletransgenic mice (MMTV-neu/MMTV-rtTA/Tet-O-Brk) for the purpose to determine whether Brk induction will accelerate the breast tumor occurrence in MMTV-neu transgenic mice. Through studies of gain-of-function of Brk in MMTV-neu transgenic mice, we will gain insight into whether Brk functions to facilitate and/or is required for HER2/neu-induced tumorigenesis in mice, important questions that have yet to be answered through a defined genetic model.

MATERIALS AND METHODS

Animals and tissues

Brk transgenic mice (Tet-O-Brk) were engineered by inserting the coding sequence of wild type Brk, constitutively active Brk (Y447F) or kinase dead Brk (K219M) downstream of the tet operator in TMILA plasmid (a gift of Randy Johnson) with an IRES-Firefly Luciferase sequence located down-stream of Brk. Founder lines provided by M.D. Anderson Genetically Engineered Mouse Facility (GEMF) were generated by

injecting the linearized construct into fertilized oocytes harvested from superovulated FVB mice.

Brk transgenic mice (Tet-O-Brk) (constitutively active Brk genotype and wild type Brk genotype) were crossed to MMTV-rtTA (MTB) mice (FVB) to generate the MMTV-rtTA/Tet-O-Brk doxycycline-inducible transgenic mice. MMTV-rtTA/Tet-O-Brk female mice of each constitutively active Brk (Y447F) genotype were administered doxycycline in their drinking water beginning at 8 weeks of age and were monitored for mammary tumors once weekly.

Dr. Randy Johnson, a member of my advisory and supervisory committee, has obtained the MTB transgenic line of mice and then TMLA plasmid from Dr. Chodosh's laboratory at University of Pennsylvania. These resources are available to me through collaboration of Dr. Johnson and my advisor, Dr. Fan.

MMTV-rtTA/Tet-O-Brk mice of wild type Brk genotype were further crossed with MMTV-neu mice to generate MMTV-neu/MMTV-rtTA/Tet-O-Brk doxycycline-inducible transgenic mice. Transgene expression was induced by replacing normal drinking water with water containing 5% sucrose and 2mg/ml doxycycline beginning at 8 weeks of age and the mice were monitored for mammary tumors once weekly. Mice were sacrificed when its possessing tumors reach the size of 20 mm in diameter. The tumors and organs were examined at necropsy and harvested for further experimentation. Tumor multiplicity was calculated as the average number of tumors per animal visualized macroscopically at necropsy.

Transgenic mice were housed under conventional conditions with a 12 hr light/dark cycle and access to food and water ad libitum. Induced animals were administered doxycycline 2 mg/ml (Sigma) in their drinking water which was replaced weekly. Animals were inspected for tumors, and existing tumors were measured weekly. At the indicated times of sacrifice, animals were killed by CO₂ asphyxiation and tissues were either frozen in -80⁰C for protein analysis, or fixed in 4% formaldehyde for morphological and immunohistochemical analysis.

Luciferase assay and western blot

Frozen mammary gland tissue was analyzed using the Luciferase Assay System (Promega) per manufacturer's instructions. Tissue was dounced in 1 ml Passive Lysis Buffer (Promega) and lysates were centrifuged at 4 ⁰C for 20 min at 14,000 rpm. 20 ul lysate was mixed with 20 ul Luciferase Assay Substrate (Promega) and luciferase activity was read immediately in a Fluostar Omega luminometer (BMG labtech). Luciferase activity levels were normalized to total protein levels as determined by Lowry Protein Assay (BioRad). Simultaneously 100 ug protein of the lysate were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred onto nitrocellulose by Western blotting, and probed with Brk antibody and horseradish peroxidase-labeled secondary antibody. The signals were visualized with an enhanced chemiluminescence detection kit (GE Healthcare).

In vivo bioluminescence imaging

Mice were anesthetized with 2.5% isofluorene and administered an intraperitoneal injection of 150 mg/g D-Luciferin (Xenogen, Hopkinton, MA). Ten minutes after

injection, the animals were placed within the Xenogen CCD imaging apparatus and images were acquired using the Xenogen IVIS 100 system and analyzed using the Living Image Xenogen Software.

Whole mount staining and histology

Number 3 or 4 mammary glands were mounted on glass slides, fixed in 4% paraformaldehyde for 2hr at 4 °C. Glands were rinsed with 1X PBS three times and stained in a filtered solution of 0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate for overnight at room temperature. Glands were then dehydrated sequentially through 75%, 95%, and 100% ethanol for 15 min each, then cleared in xylene overnight, then defatted and stored in methylsalicylate. For histological analysis, fixed glands in 4% paraformaldehyde were blocked in paraffin, sectioned, and stained with hematoxylin and eosin.

Genotyping

All the mice were labeled with toe clipping method. The cut toes of mice were collected and lysated in 200ul Direct PCR lysis buffer (lysis reagent for genotyping using crude lysates) (Cat#102-7, VIAEN, LA, CA) with 20mg/ml proteinase K. After incubation at 55 °C overnight, the tissue were inactivated at 100 °C for 10 minutes and then centrifuged at max for 15 minute to remove pellets. 1-2 ul supernatant was used to detect Brk, MTB or HER2 expression through PCR.

RESULTS

Generation of MMTV-rtTA/Tet-O-Brk doxycycline-inducible transgenic mice

To generate MMTV-rtTA/Tet-O-Brk bitransgenic mice containing a Brk cDNA that is controlled by a novel doxycycline-inducible system for mammary epithelium-specific transgene expression (MMTV-rtTA/Tet-O-Brk), firstly cDNA of wild-type Brk, kinase-dead Brk (Brk-K219M), or constitutively active Brk (Brk-Y447F) were subcloned into the TMILA plasmid. Inclusion of the Brk mutants in the study will allow us to assess the role of Brk kinase activity in potentiating breast tumor formation-Brk may function as an adaptor molecule independent of its kinase activity. The Tet-O-Brk transgenic mice was generated with assistance from the Genetically Engineered Mouse Facility (GEMF) at M.D. Anderson Cancer Center. Founders are identified by PCR-based screening of genomic DNA from tail biopsy specimens for the presence of the transgene. All transgenic lines are created and maintained on an inbred FVB/N strain background to match with the FVB/N background of MMTV/neu transgenic mice (Jackson Laboratory) and MTB mice. (Fig.20) Three-five founders (G0) for each Brk construct were obtained from GEMF of M.D. Anderson Cancer Center and were further crossed with the MTB line to obtain the bitransgenic mice (MMTV-rtTA/Tet-O-Brk) (G1). Transgene expression was induced in G1 mice starting from age of 8 weeks by replacing normal drinking water with 5% sucrose containing 2mg/ml doxycycline, the G1 mice in the control group were fed with 5% sucrose water. After 4 day of induction, fresh mammary glands were taken and lysated for luciferase assay and western blot for examining Brk expression. Two (319 and 322) of five Brk-Y447F lines (CA) and two (483 and 497) of five wild type Brk lines (WT) exhibited maximal induction of luciferase activity and had the expression of Brk as detected by western blot in mammary tissue in the presence of

doxycycline. However, none of the three Brk-K219M lines showed evidence of Brk induction (Table 3) (Fig. 21 A and B). At the time of 3 week and 6 week of Dox treatment, IVIS showed that luciferase activity was well induced in both Brk CA (319) and Brk WT (497) lines of bitransgenic mice (MMTV-rtTA/Tet-O-Brk). (Fig. 21 C)

Figure 20. Generation of MMTV-rtTA/Tet-O-Brk doxycycline-inducible transgenic mice

cDNA of wild-type Brk, kinase-dead Brk, and constitutively active Brk were subcloned into the TMILA plasmid. Brk transgenic mice were generated by injecting the linearized constructs into fertilized oocytes harvested from superovulated FVB mice. The identified transgenic mice were further crossed with MMTV-rtTA transgenic mice (MTB) to generate MMTV-rtTA/Tet-O-Brk doxycycline-inducible double transgenic mice. The double transgenic mice were fed with 2mg/ml doxycycline containing water, expression of rtTA specifically in the mammary tissue binds to the tet-o promoter of Brk transgene and initiate the Brk gene expression in the mammary gland.

Figure 20

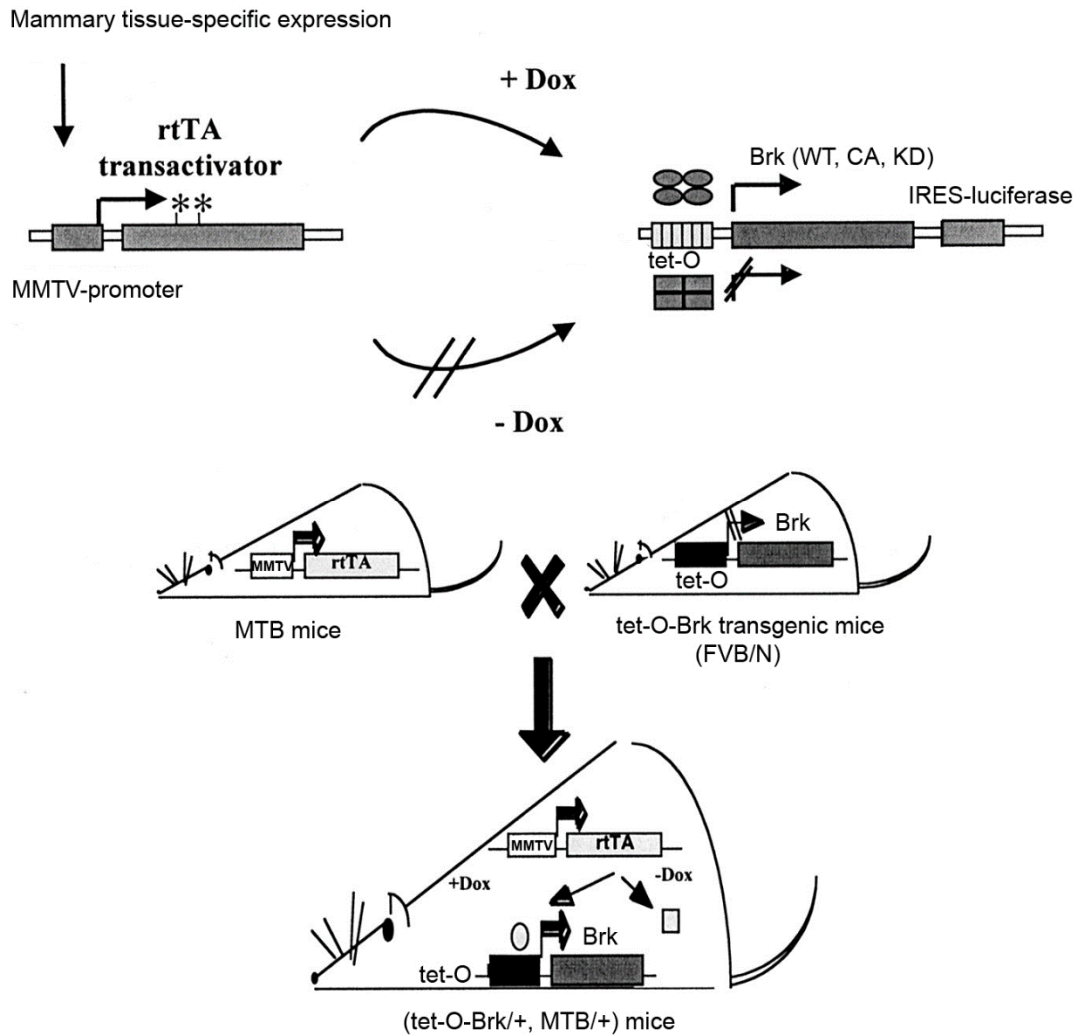


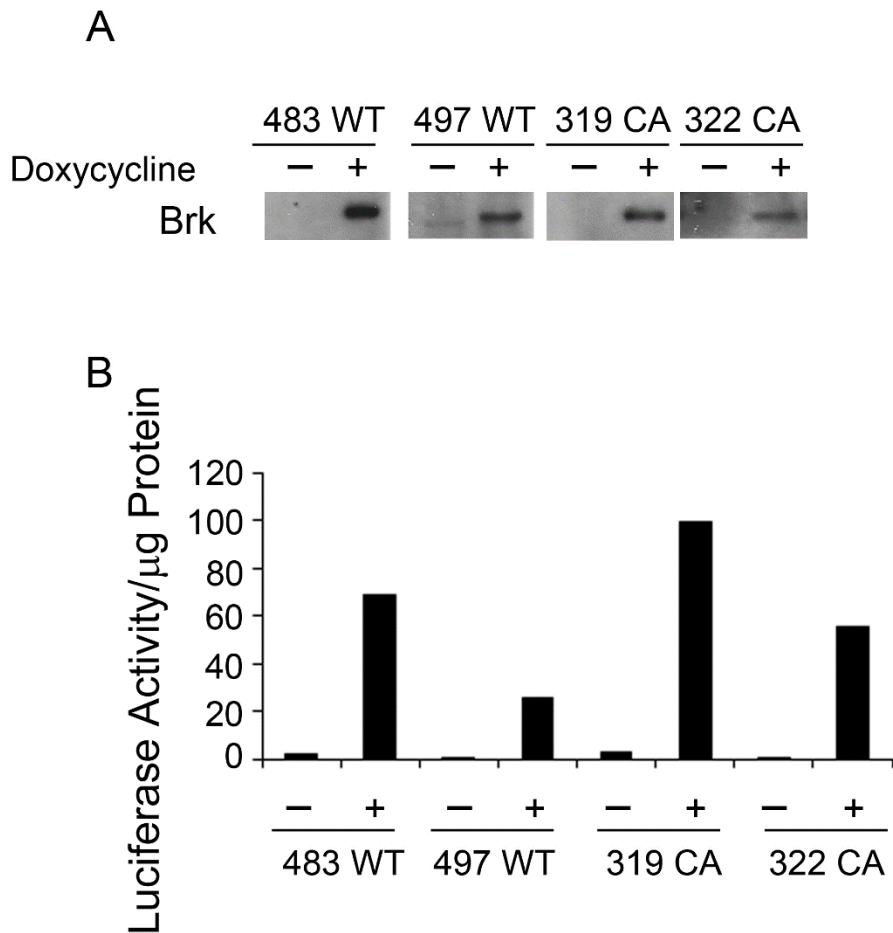
Figure 21. Induction of Brk expression in MMTV-rtTA/Tet-O-Brk transgenic mice

A). Western blot was used to identify the induction of Brk protein expression in the mammary tissue of different MMTV-rtTA/Tet-O-Brk transgenic mouse lines which were maintained on doxycycline for 4 days.

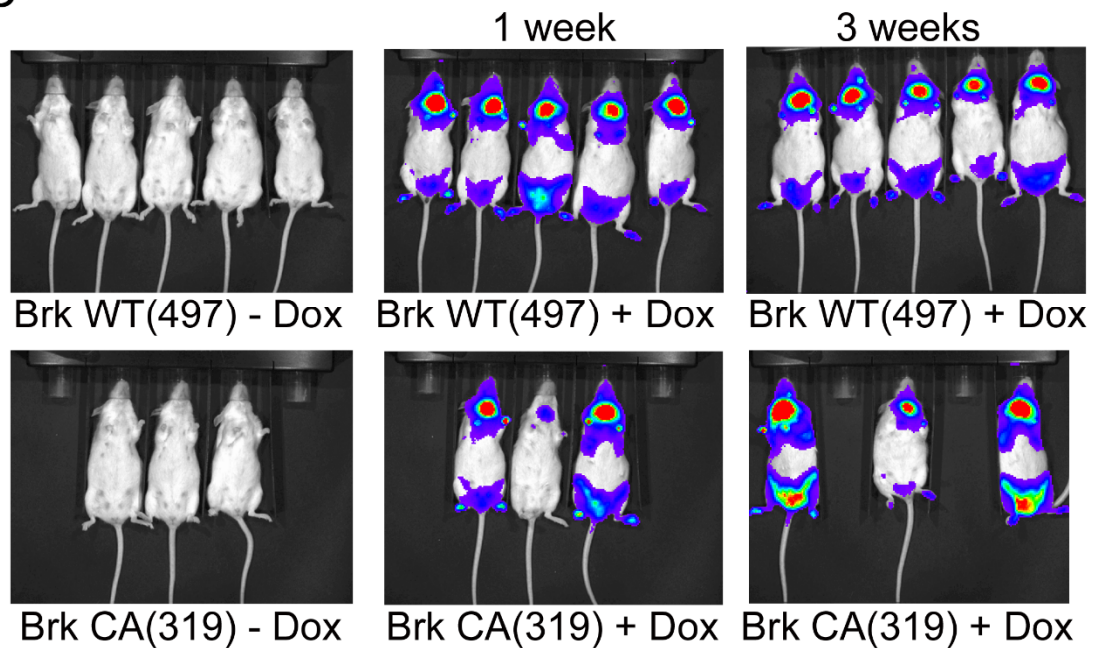
B). Luciferase activity assays were performed on mammary tissue of different MMTV-rtTA/Tet-O-Brk transgenic mouse lines maintained on doxycycline for 4 days. Assays were performed in triplicate and relative light units were normalized to total protein levels.

C). Bioluminescent images of luciferase signal present in MMTV-rtTA/Tet-O-Brk transgenic mouse body of both WT (497) and CA (319) lines after doxycycline induction for 1 week and 3 weeks.

Figure 21



C



Induction of Brk expression in MMTV-rtTA/Tet-O-Brk CA transgenic mice

We randomly divided 52 females of Brk-CA (MMTV-rtTA/Tet-O-Brk) bitransgenic mice (319) equally to two groups. One group of mice received 5% sucrose plus 2mg/ml doxycycline (Dox) water for continuous Brk induction and the other group of mice received 5% sucrose water only as the control, all the water was changed weekly. As additional control group, a cohort of MTB females (n=10) were treated with 2mg/ml Dox too to determine the effects of Dox alone on mammary glands.

IVIS showed that luciferase activity was well induced at the time of 1 week and 3 week of Dox treatment (Fig.21 C), the intensity of luciferase activity did not decrease even on 7 month of treatment although there is a little reduction on 15 month of treatment. (Fig. 22 A and B) To accelerate the tumor occurrence, all the mice were breed 2-3 times after one month of induction. During the time of mating, pregnancy and nursing, all the

mice still were maintained with special water. The experiment was ended at the time of Dox induction for 15 months with all the mice sacrificed. Till then, in the control group, 3/26 mice died for unknown reason at 3 moth, 3 month and one year of treatment respectively; 1/26 mice had severe dermatitis and was sacrificed at 7 month of treatment; 1/26 mice died for exophthalmos at the time of one year treatment and 1/26 mouse died for paralysis at the time of 14 month treatment; 2/26 mice had randomly spontaneous tumors by the anus and inside liver, the mouse with tumor near anus was sacrificed at 8 month of treatment and mouse with liver tumor was sacrificed at 15 month of treatment. In contrast, in the experimental group, there were also 3/26 died for unknown reason at 3 month, 5 month and 8 month of treatment; 1/26 mouse died at the time of 5 month treatment and the blood clots was found beside the mammary gland; 2/26 mice had randomly spontaneous cervix tumors and 2/26 had mesentery swelling which were sacrificed at the time of 15 month of treatment, and 5/26 mice had severe dermatitis which were sacrificed at 6 month, 8 month, 10 month, 10 month and 13 month of treatment upon request, however, all these mice neither had visible breast tumor nor mammary gland malignant change in the histological level. The survival curve was drawn in Figure 22. (Fig.22 C)

Figure 22. Induction of Brk expression in MMTV-rtTA/Tet-O-Brk CA transgenic mice

Bioluminescent images of luciferase signal present in MMTV-rtTA/Tet-O-Brk transgenic mouse body of Brk CA(319) after doxycycline induction for 7 months (A.) and 14 months (B.).

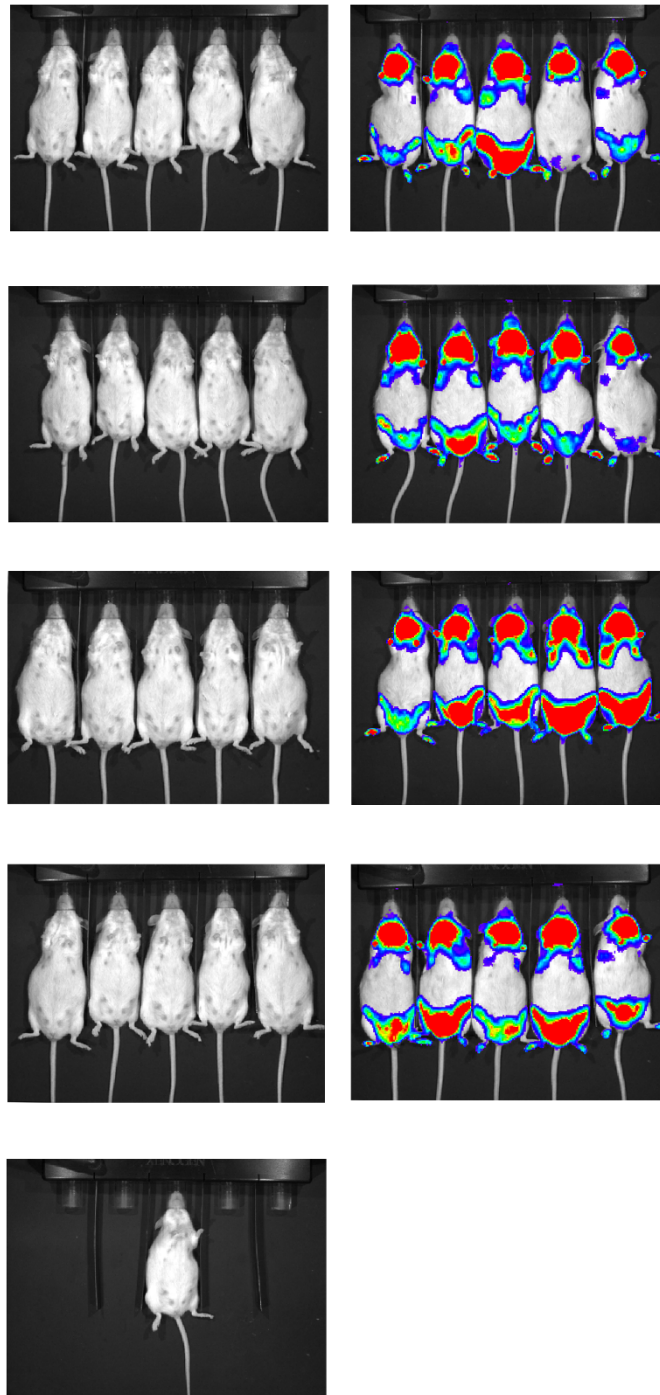
Figure 22

A

7 months of induction

Brk CA (319)/MTB- Dox

Brk CA (319)/MTB+Dox

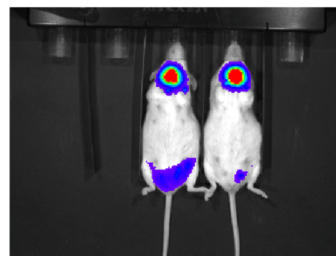
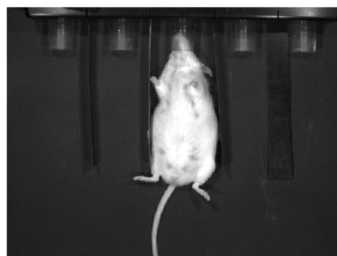
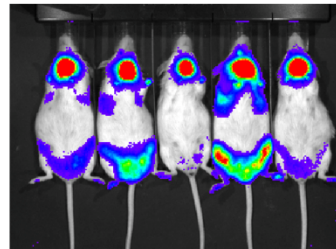
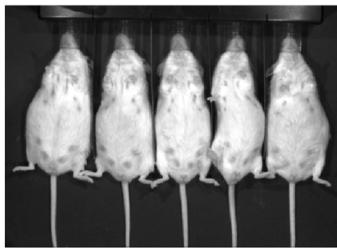
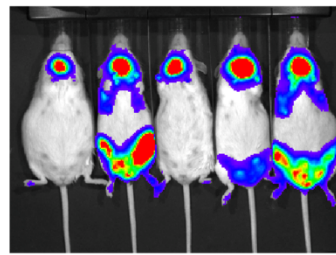
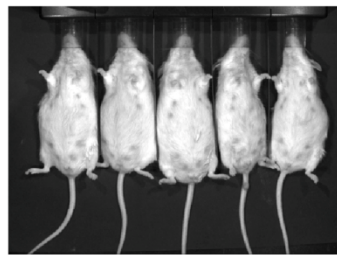
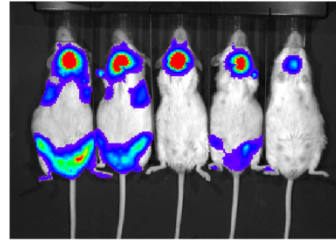
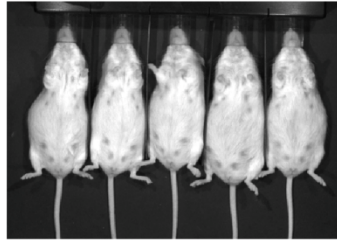


B

14 months of induction

Brk CA (319)/MTB- Dox

Brk CA (319)/MTB+Dox



Generation and analysis of MMTV-neu/MMTV-rtTA/Tet-O-Brk triple transgenic mice

The MMTV-neu transgenic mice are known to develop focal mammary tumors that first appear at 4 months, with a median time to tumor development of 205 days [193]. Both virgin and breeder mice develop tumors that arise as foci in hyperplastic, dysplastic mammary glands [193]. To determine whether sustained induction of transgene expression of Brk expedites tumor development in the mammary epithelium in MMTV-neu transgenic mice, MMTV-rtTA/Tet-O-Brk mice (G1) (wild type Brk genotype) were crossed with MMTV-neu transgenic mice to generate triple-transgenic mice (MMTV-neu/ MMTV-rtTA/Tet-O-Brk mice) that contain the MMTV-neu, MTB, and Tet-O-Brk transgenes (Fig. 23). A cohort of 60 triple-transgenic females of 483 line were treated with (n=30) or without (n=30) doxycycline beginning at 8 weeks of age and were closely monitored for tumor development by palpation weekly. Spontaneous breast tumor developed in a stochastic manner in both groups with median tumor latency time (T50) of 214 days in control group and 220 days in experimental group. There was not statistical difference in the tumor free curve between these two groups as analyzed by Log rank test (Fig.24 A). Once palpable tumors have formed, tumor growth was measured in two dimensions with calipers. When the mammary tumors reach a diameter of 10-15 mm, the mice was sacrificed and the tumors were collected. I compared the tumor size, tumor weight and tumor multiplicity between these two groups and did not find significant difference either beside the fact that there was no difference in the survival curve and tumor harboring survival curve (Fig.24 B, C, D, E and F). Tumor tissue, normal mammary glands and metastatic organs such as lung and liver were archived for immunohistologic and pathologic analysis. At the time of 8 month induction, IVIS

showed that in the control group there was no luciferase activity and in the experimental group only 6/14 mice showed luciferase activity. (Fig 25 A) Simultaneously, two mice from the control group and three mice from the experimental group were randomly chosen to detect Brk protein expression both in the normal mammary gland (G) and breast tumor (T) through western blot. We only detected Brk protein expression in the normal mammary gland but not in the tumor tissue of one mouse from the experimental group (Fig 25 B). The expression of Brk was not observed in the glands and tumor tissues of the mice from the control group and the other two mice from the experimental group. In addition, compared with the expression level in the mammary glands, HER2 protein level in the tumor tissue among all the mice increased dramatically, suggesting that overexpression of HER2 may suppress Brk protein expression according our previous study.

Figure 23. Generation of MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mice

Wild type MMTV-rtTA/Tet-O-Brk transgenic mice were crossed with MMTV-neu transgenic mice to generate MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mice which has mammary tissue specific expression of rtTA and HER2. In the presence of doxycycline, rtTA induced Brk transgene expression in the mammary gland.

Figure 23

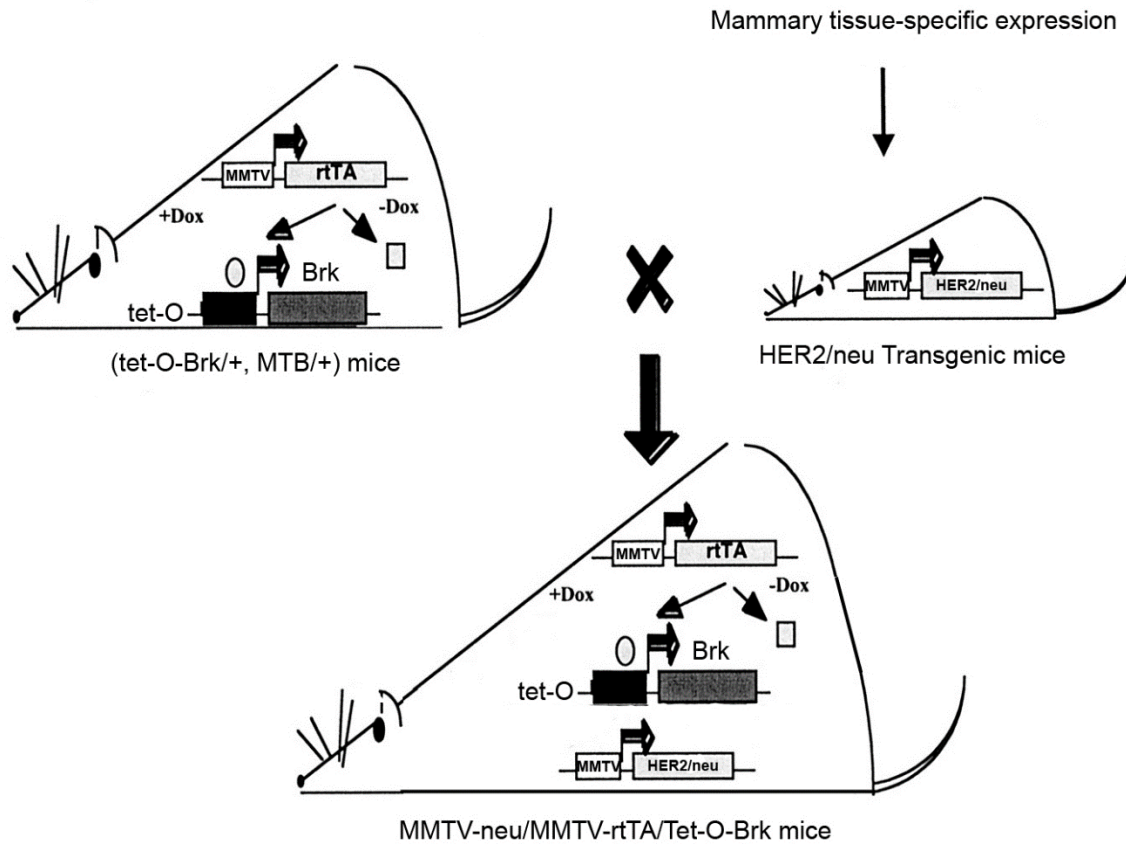


Figure 24. Induction of Brk does not alter breast tumor development induced by HER2/neu in MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mice

A). The number of tumor-free days for each group of animals are shown. Brk wild type 483 line triple transgenic mice were maintained with 5% sucrose water in the control group (n=30) and 5% sucrose plus 2mg/ml doxycycline water in the experimental group (n=30) for 350 days. T50 represents the time point when 50% of mice in that group developed tumors.

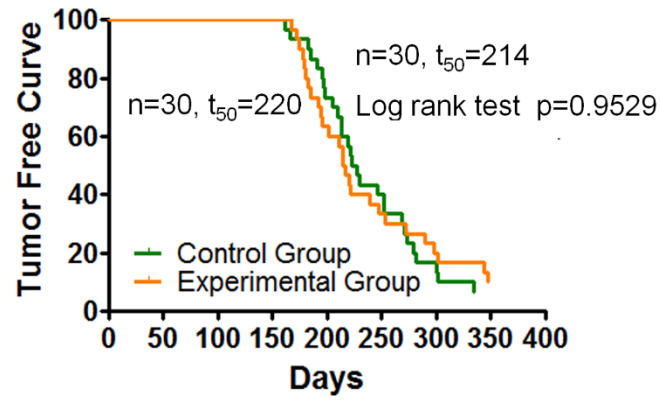
B). Kaplan-Meier survival curves for each group of animals are shown. 20 mice in each of the control group and experimental group were calculated for survival curve. Brk wild type 483 line triple transgenic mice were maintained with 5% sucrose water in the control group and 5% sucrose plus 2mg/ml doxycycline water in the experimental group for 350 days. T50 represents the time point when 50% of mice died.

C). Kaplan-Meier survival curves of mouse tumor harboring time in control group and experimental group are shown. Brk wild type 483 line triple transgenic mice were maintained with 5% sucrose water in the control group and 5% sucrose plus 2mg/ml doxycycline water in the experimental group for 350 days. T50 represents the time point when 50% of mice harboring tumor died.

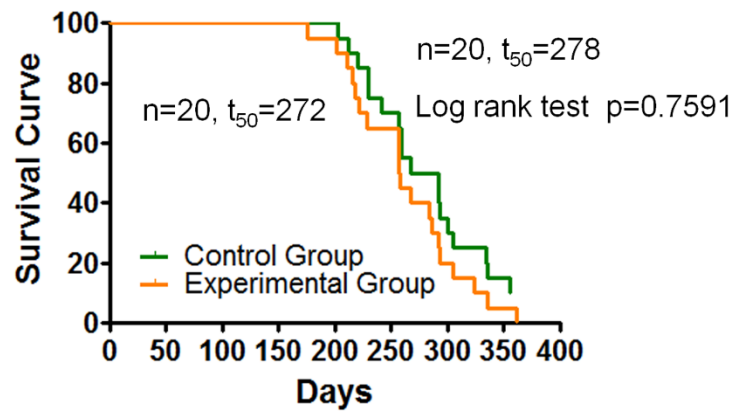
D, C, E). Mammary tumor size, weight and multiplicity are not significantly different ($P > 0.1$) in the mice of control group versus experimental group.

Figure 24

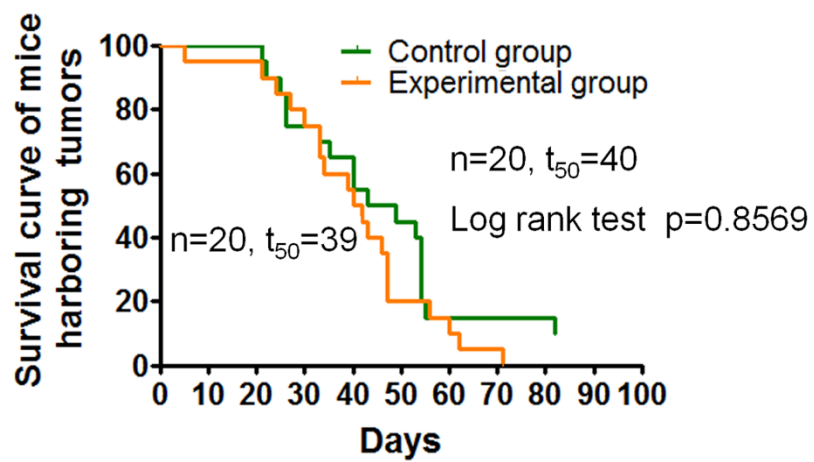
A



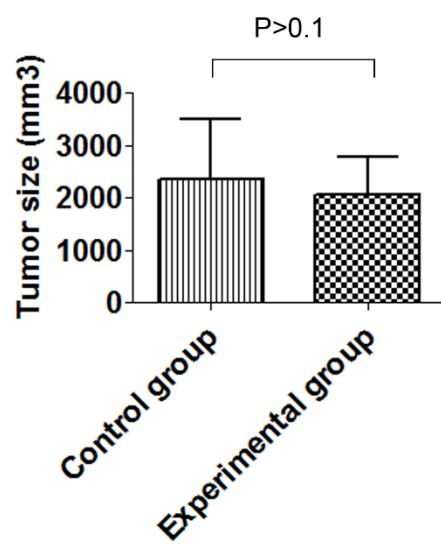
B



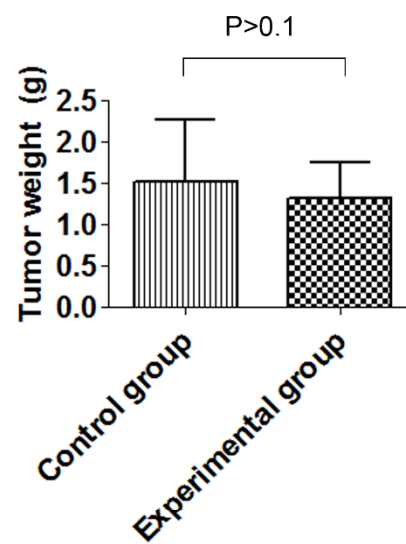
C



D



E



F

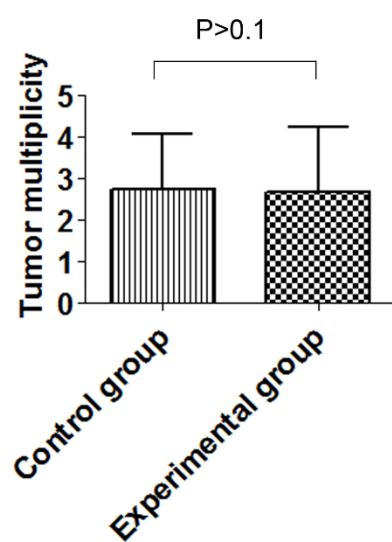


Figure 25. Induction of Brk in MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mice of wild type 483 line

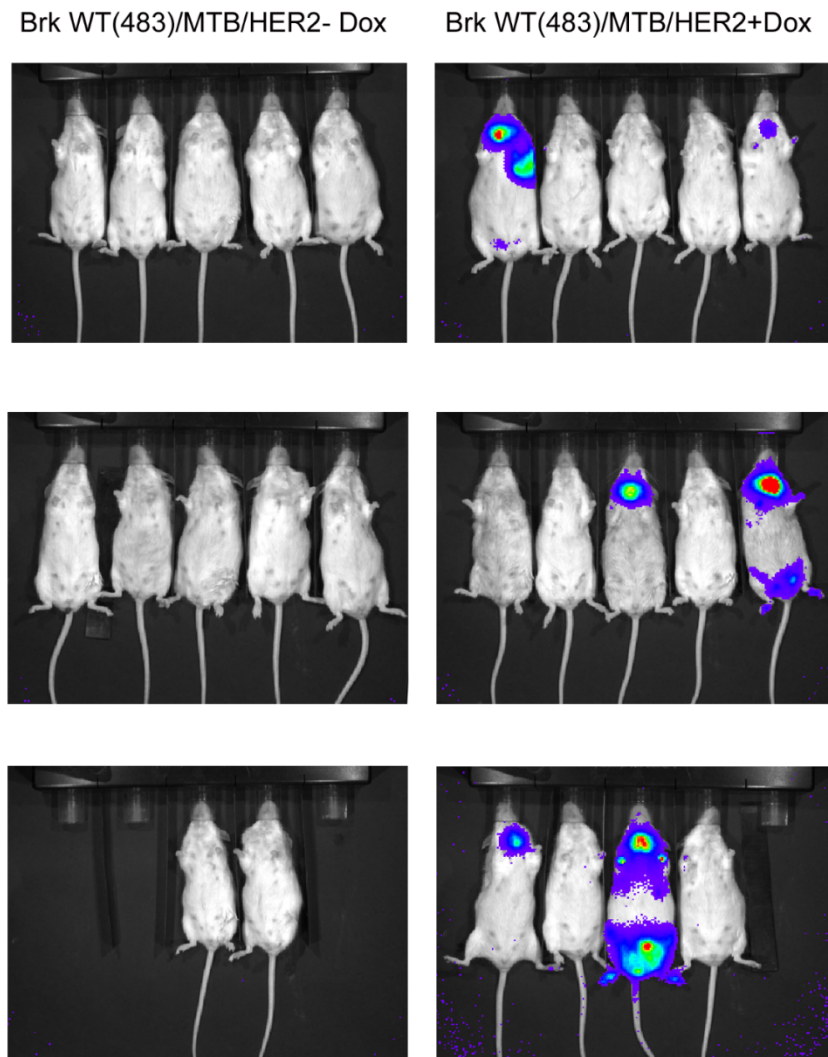
A). Bioluminescent images of luciferase signal present in MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mouse body of Brk WT (483) after doxycyclin induction for 8 months

B). Induction of Brk protein expression in normal mammary gland(G) and breast tumor tissue(T) of MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mouse WT(483) as examined by western blot after doxycycline induction for 5 months

Figure 25

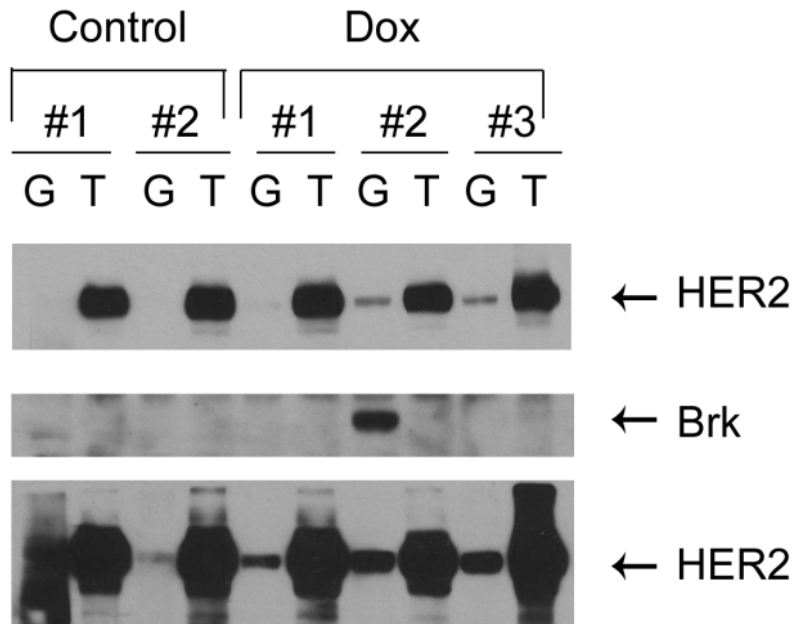
A

8 months of induction



B

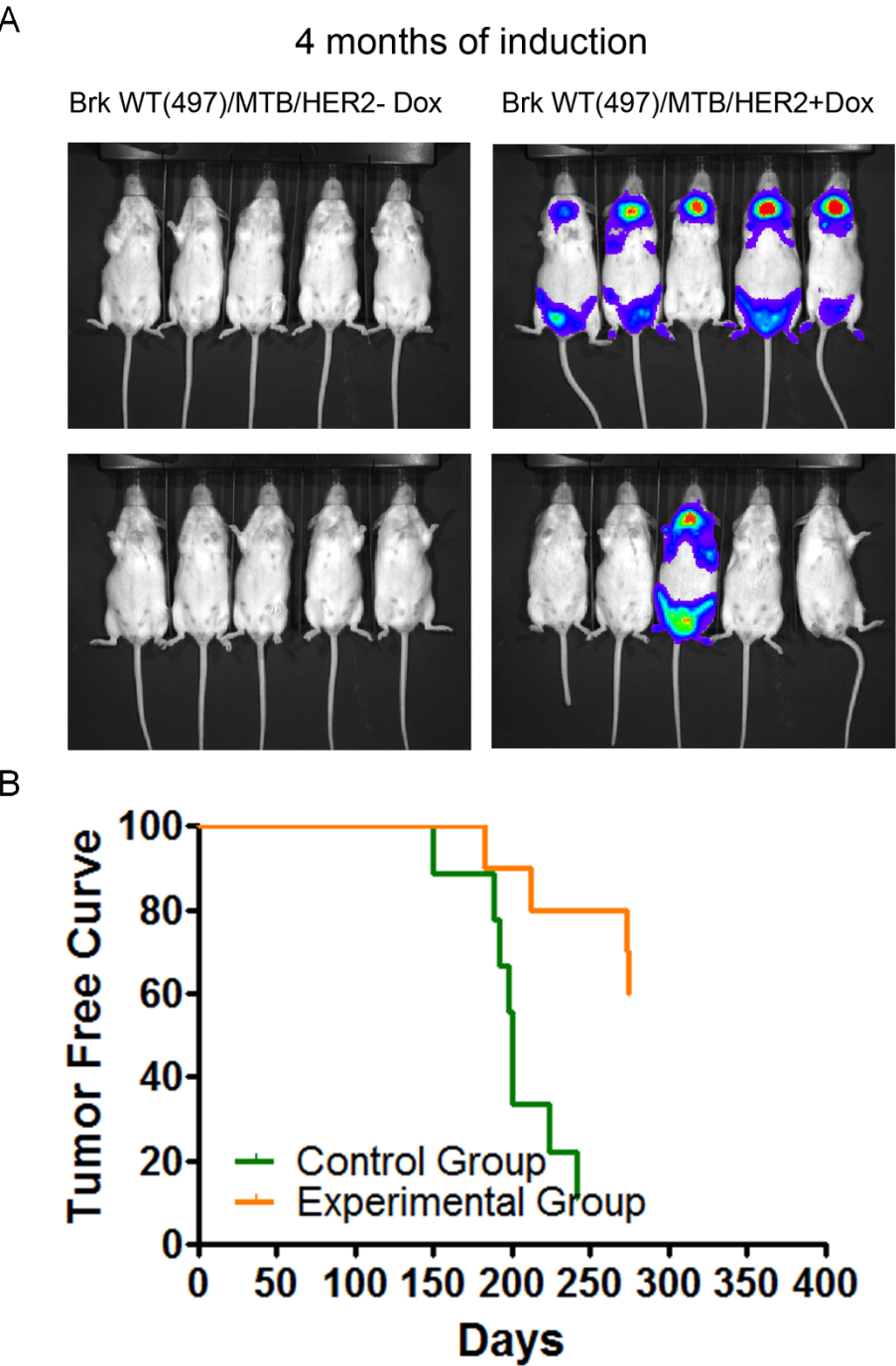
MMTV-neu/MMTV-rtTA/Tet-O-Brk



In the line of 497 triple transgenic mice, 20 females were evenly divided into control group and doxycycline treating group, and were continuously treated with or without 2mg/ml doxycycline water. Bioluminescence imaging showed that at the time of 4 month induction, 6/10 mice fed with doxycycline had luciferase activity. And luciferase signaling in the mice of control group was negative (Figure 26 A). Till the time of 8 month induction, in the control group, one mice died for unknown reason, and 8/9 mice had breast tumors and died for the disease with average tumor harboring time of 43.1 days, whereas in the experimental group, only 4/10 mice had breast tumor with 2 mice died for it at average tumor harboring time of 48 days. The tumor free curve of mice in experimental group was significantly delayed compared with that of the control group. (Figure 26B).

Figure 26. Induction of Brk in MMTV-neu/MMTV-rtTA/Tet-O-Brk triple-transgenic mice of wild type 497 line

Figure 26



DISCUSSION

In this part, we generate transgenic mouse models that inducibly express an activated form of Brk (i.e., BrkY447F) and wild type Brk in mouse mammary glands. The continuous expression of constitutively active Brk in the mouse mammary gland neither induces breast tumor nor any hyperplasia of mammary tissue through histological analysis. Co-expression of wild type Brk in mammary gland of MMTV- neu transgenic mice does not expedite breast tumor occurrence.

Expression of constitutively active Brk alone in the mouse mammary gland can not initiate breast tumor. The negative result may be due to the following reasons: i) We don't know whether Brk was really activated in the mammary gland of the transgenic mouse. Since that we transfected a human Brk CA in mouse body whether it can mediate the same signaling alterations in mouse as it does in human cell remains unknown. We may design an experiment to examine the signaling change in mammary tissue with or without Brk induction. ii) The intensity of Brk expression induction was not strong enough to initiate malignant phenotype in mammary tissue. iii) Brk is not a tumor generating gene so expression of activated Brk alone is not sufficient to induce breast tumor. iv). Brk may be involved in the metastasis of breast tumor instead of inducing breast tumor considering the gene was cloned from a metastatic breast tumor. Lange's laboratory generated the WAP-driven Brk/PTK6 transgenic mouse model in FVB/n mice too and they found that Brk has very weak role in promoting tumorigenesis[266], although they observed that WAP-driven Brk expression delayed involution of mammary gland after weaning. The exact functions of Brk during breast cancer development still need be defined.

In the 483 line of MMTV-neu/MMTV-rtTA/Tet-O-Brk triple transgenic mice we did not observe that the expression of Brk altered average latent time of breast tumor in the mouse model. Interestingly, in both 483 line and 497 line only partial mice showed the luciferase activity as examined by IVIS, and western blot detected Brk expression in mammary gland only in one of three selected mice fed with doxycycline. Furthermore, the tumor tissue of MMTV-neu mice contain significantly higher expression level of HER2 than the normal mammary, however Brk expression was detected only in the normal mammary tissue but not in the tumor tissue. The differential expression pattern suggests that Brk expression may be inhibited by the over-expression of HER2. Our previous data found that there is complementary interaction between HER2 and Brk. It is possible that overexpression of HER2 in the breast tumor of triple transgenic mouse suppress Brk expression, although the underlying mechanism about how HER2 inhibits Brk expression is largely unknown.

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